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14. ABSTRACT

To benefit military veterans with amputations who suffer skin problems on their amputation stumps, this proposal describes mechanistic studies to pave the way for novel methods of improving skin barrier function at the residual limb-prosthetic interface.
Signaling systems in skin will be modulated to increase barrier function, attenuate irritant dermatitis, and characterize the underlying signaling mechanisms so that they can become better targets for treatment.

Progress in year 2 of the funding period is described in this Annual Progress Report.
We maintained all the necessary regulatory approvals from the Durham VA, Duke University IRB and the DoD to conduct the human experimentation. We set up experiments in primary skin cells for mechanical stress, which we found disrupts skin barrier function. We also found that activation of ion channel TRPV4 can re-normalize barrier function of the skin that has been disrupted by mechanical stress. We also found this particular pattern for keratinocytes' regulatory volume decrease, as a surrogate of their capability to moisturize.

15. SUBJECT TERMS
TRP ion channel, epidermis, keratinocyte, mechanical stress, epithelial stress response

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Introduction

The objective of our proposal is to benefit military veterans with amputations who suffer skin problems on their amputation stumps. To fulfill the objective, this proposal describes mechanistic studies to pave the way for novel methods of improving skin barrier function at the residual limb-prosthetic interface.

Rather than attempting to enhance the functions of barrier-enhancing proteins, filaggrin (structural protein in upper epidermal layers) and aquaporins (water channel) by individual targeting, we thought it an attractive (and testable) idea, whether targeting of any of the epidermal transient receptor potential ion (TRP) ion channels could lead to **improved** barrier function and thus better moisturization, thus less irritation, injury and pain. This would be accomplished by affecting both, filaggrin and aquaporin. In keeping with our reasoning, recent insights on activation of TRPA1 and TRPV4 in the skin are supportive of our concept. However, an obstacle toward improved insight is that mammalian skin was not studied in response to mechanical stress in order to wear down the barrier, so that its more rational repair, by targeting specific signaling mechanisms of the epidermis, can be assessed.

Hypothesis

Therefore, the central hypothesis of our proposal is that Ca^{++} influx into epidermal keratinocytes – mediated via TRP ion channels that we intend to activate specifically - controls moisturization and barrier function of the skin by critically influencing filaggrin- and aquaporin function in a cell-autonomous manner, and that activation of these TRP channels can be helpful for repairing a compromised barrier which is caused by injurious mechanical stress.

Specific Aims

(1) to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal human skin as it is subjected to mechanical stress.

(2) to assess skin barrier function and moisturization in epidermal cultures derived from military veterans with amputations and stump skin irritation, and to determine the epidermis' response to activation of TRP channels.

Keywords

Amputation stump, irritant dermatitis, epidermis, skin barrier function, TRP channel, aquaporin (AQP) channel, UVB, UVA

Accomplishments

Stress-testing of human artificial skin – accomplished consistent strain >0.5
Pertains to both following subtasks
<u>Specific Aim 1, Subtask 3</u>: *to assess skin barrier function and moisturization parameters in response to modulation of TRPV4 and TRPA1 in normal skin, using UV-light activation*
<u>Specific Aim 1, Subtask 4:</u> *to assess Filaggrin metrics by Western blotting, immunolabeling, treat human organotypic skin as in Subtask 1*

In an attempt to optimize application of increased strain, we repeated strain testing, and were able to obtain strains above 0.5, reliably, but still not reliably to the level of 0.6 (Figure 1).
We used human artificial skin as a organotypic human skin substrate, and exerted compression with our bioreactor, evoking the depicted strain.

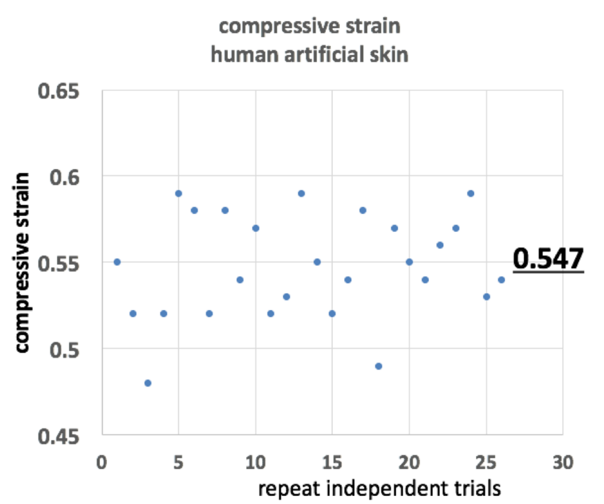

Fig. 1: Strain >0.5 in human artificial skin by bioreactor compression

Stress-testing of human artificial skin – barrier improvement by exposure to UVB pertains to
__Specific Aim 1, Subtask 3__: *to assess skin barrier function and moisturization parameters in response to modulation of TRPV4 and TRPA1 in normal skin, using UV-light activation*

EpiDermF were grown in culture. We applied mechanical stress at a strain of 0.5, using our modified Flexcell Bioreactor Compression system. We operated it at 1/sec sinusoidal strain for 1h, followed by 1h rest, for a total of 12h.
We applied long-term mechanical stress testing, over 10d, to assess the effect of TRPV4 activation using UVB with the increased exposure protocol (200kJ/cm^2 for 5 min, 10 min preceding mechanical stress). By doing so, we were able to partially rescue the diminished and diminishing capacitance, again encountering some increased variation for the UVB response (Fig. 2).

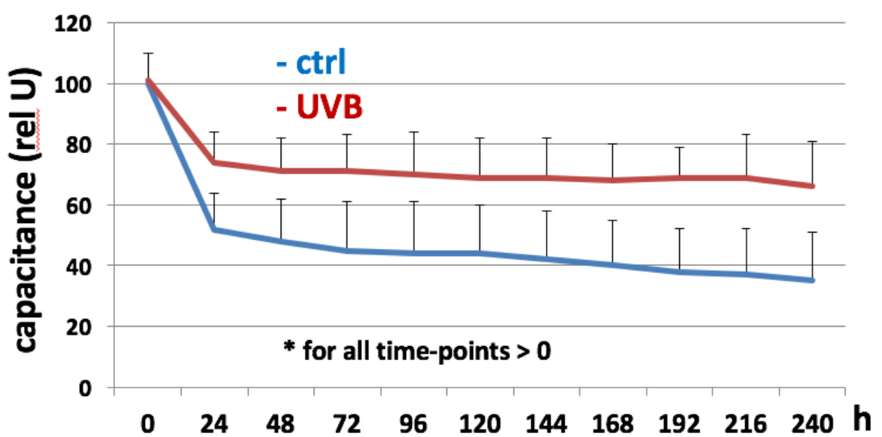

Fig. 2: Long-term mechanical compression-stress, to human artificial skin, attenuates capacitance, as a surrogate of barrier function, rescue by application of UVB, shown previously to activate epidermis TRPV4 and increase capacitance. Note mechanical stress here at 0.5 strain level for each application (daily). Experimental n per group n=6; student's t-test for all p-values, which were $p<0.05$ for all time-points starting at 24h.

Measurement of skin capacitance in culture – dependence on TRPV4
pertains to
<u>**Specific Aim 1, Subtask 3**</u>: *to assess skin barrier function and moisturization parameters in response to modulation of TRPV4 and TRPA1 in normal skin, using UV-light activation*

We were able to increase skin capacitance using UVB, but had to increase the UVB-exposure. There remained considerable variation, still, but the increase was significantly attenuated by using TRPV4 blocking molecules (Figure 3).
We used human artificial skin as human skin organotypic substrate, UVB activation of TRPV4 was implemented at energy levels of 200kJ/cm^2 for 5 min, 10 min later followed by measurement of capacitance, a valid surrogate for barrier function.

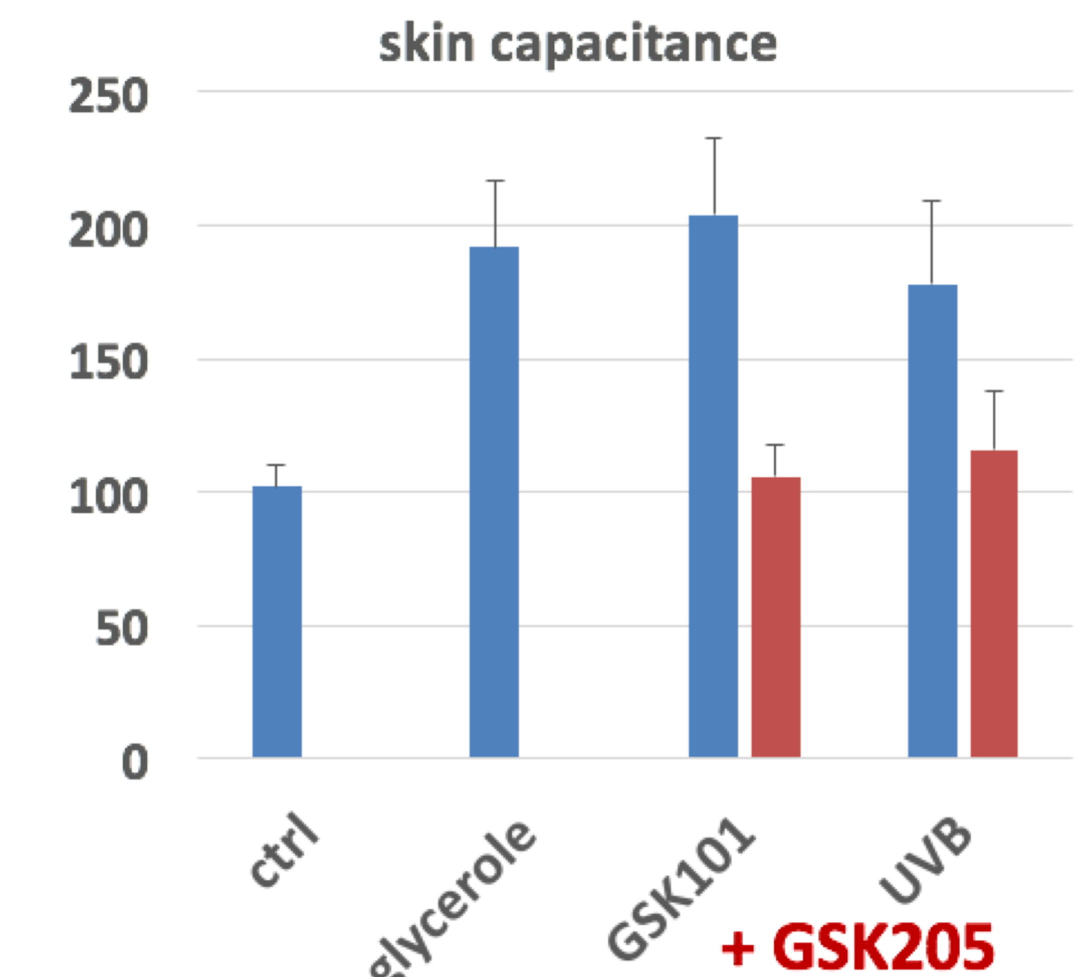

Fig. 3: TRPV4-dependent increase in skin capacitance in human artificial skin

Diminished Filaggrin expression in mechanically-stressed artificial human skin pertains to

<u>Specific Aim 1, Subtask 4:</u> *to assess Filaggrin metrics by Western blotting, immunolabeling, treat human organotypic skin as in Subtask 1*

We detected a significant decrease of filaggrin, both at the mRNA and protein level in response to mechanical stress for 28 h, experimental approach as in Fig. 2.
We then attempted to rescue of diminished filaggrin expression, a telling surrogate of skin barrier dysfunction, by activation of TRPV4 with specific activator GSK101 (100nM). Initial results were encouraging, yet continuous attempts were not met with consistent success. We are currently working on establishing the most effective protocol at stimulating TRPV4 in order to obtain the most effective barrier repair.

Regulatory volume decrease (RVD) of epidermal keratinocytes depends on TRPV4 pertains to

<u>Specific Aim 1, Subtask 7:</u> *To dissociate keratinocytes from human organotypic skin, subject dissociated cells to hypotonic cell swelling; determine Ca^{++} dependence of regulatory volume decrease by application of specific TRP channel inhibitors for channels TRPV1, TRPV3, TRPV4 and TRPA1.*

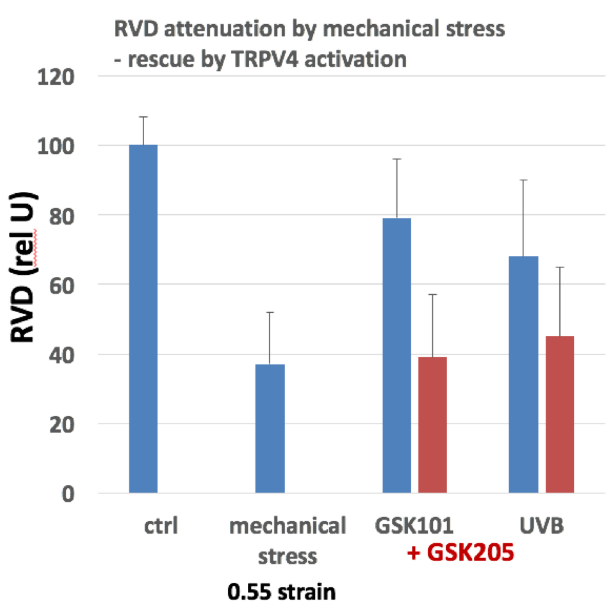

Fig. 4: Attenuation of RVD of cultured human epidermal keratinocytes in response to mechanical stress, rescue by chemical and UVB-mediated activation of TRPV4 (red bars = elimination of rescue by adding TRPV4-selective blocking molecule, GSK205 (10μM)). Increase over mechanical stress when using GSK101 or UVB at $p<0.05$ (one-way ANOVA, post-hoc Tukey testing), not different when adding GSK205. Experimental n = 8-10 per group.

In 2D-tissue culture of keratinocytes, derived from EpidermF, we were able to partially rescue the attenuation of RVD after mechanical stress (0.55 strain) by using UVB to activate TRPV4. This partial rescue could be inhibited significantly by TRPV4-selective blocking compound, GSK205 (Figure 4). The impact of 0.55 strain over 0.5 strain was significantly more accentuated (not shown).

Impact

• recorded consistently accomplished strain levels of 0.55 with our compression apparatus, using human artificial skin
• demonstrated rescue of diminished capacitance of human artificial skin, caused by long-term mechanical stress evoked by daily injurious compression, by activation of TRPV4 channels using UVB exposure
• demonstrated increased skin capacitance by TRPV4 activation in human artificial skin via exposure to UVB
• demonstrated up-regulation of TRPV4 and down-regulation of filaggrin in mechanically-stressed skin
• demonstrated that TRPV4 activation using UVB radiation repairs impairment of regulatory-volume-decrease (RVD) caused by injurious mechanical compression at a 0.55 strain rate

Changes/Problems

Re attenuation of filaggrin expression by mechanical stress. Documenting its rescue by activation of TRPV4 continues to be of a certain level of a technical difficulty. However, we did manage to overcome other recalcitrant/refractory problems during the award period, and are therefore optimistic that we will successfully overcome this hurdle.
UVB activation of TRPV4 appears to work reliably only at increased exposures so that a lower-exposure regime will have to be identified so that the risk of UVB-mediated short- and long-term damage to the integument can be contained, when considering the translational application of UVB to amputees' stump skin.
<u>Human experimentation</u>
Challenges in recruiting human subjects have remained through y3.
To resolve, we conferred again with Dr Hall. He is more certain that the paucity of amputation stump irritant dermatitis in military personnel is likely due to the improved quality of artificial limbs. However, Dr. Hall also sounded a note of (cautious) optimism to be able to finally get to recruiting the needed number of amputees because of his increased time-slots in clinics within the VA system, and because of a wider and more reliable network of referring providers whom he had now keyed in into referring amputees with stump irritant dermatitis.

Products

At the time of writing, findings as listed under "*Impact*" are processed at the Duke University Office of Licensing and Ventures **for consideration for patent filing**.

Support from this grant forms an important basis for ongoing and pro-actively pursued considerations to **form a start-up company**, a spin-off of the Liedtke-Lab at Duke University. This aspiration is also strongly supported by the Duke University Office of Licensing and Ventures, by the PI's Department of Neurology, by the North Carolina Biotechnology Center (NCBC), and by the Harrington Discovery Institute of University Hospitals in Cleveland OH.

The following **papers** were published

Chen Y, Fang Q, Wang Z, Zhang JY, MacLeod AS, Hall RP, **Liedtke WB**. TRPV4 ion channel functions as a pruriceptor in epidermal keratinocytes to evoke histaminergic itch. J Biol Chem 2016, 291:10252-62

Kanju P, Chen Y, Lee W, Yeo M, Lee SH, Romac J, Shahid R, Fan P, Gooden DM, Simon SA, Spasojevic I, Mook RA, Liddle RA, Guilak F, **Liedtke WB.** Small molecule dual-inhibitors of TRPV4 and TRPA1 for attenuation of inflammation and pain. Sci Rep 2016, June 1;6:26894. doi: 10.1038/srep26894

Kanju P, **Liedtke W**. Pleiotropic function of TRPV4 ion channels in the central nervous system. Exp Physiol. 2016 Oct 4. doi: 10.1113/EP085790

Re paper Chen et al.
This paper was accompanied by a Duke University Press Release
https://today.duke.edu/2016/03/itchy
which was picked up by the National Institutes of Health
http://www.niams.nih.gov/news_and_events/Spotlight_on_Research/2016/ion_channel_itch.asp
and news-media world-wide, e.g.
http://www.dailymail.co.uk/news/article-3492763/Time-stop-head-scratching-Scientists-step-close-finding-cure-itching-discovering-skin-protein-causes-irritation.html

Re paper Kanju et al in *Scientific Reports*
The paper was accompanied by a Duke University press release.
https://today.duke.edu/2016/06/doublepain
It generated a lot of public attention, amongst others an interview with National Public Radio (NPR), see also
http://www.wfdd.org/story/duke-researchers-discover-new-class-pain-reliever,
and a widely-accessed posting in the "*Daily Beast*",
http://www.thedailybeast.com/articles/2016/06/01/these-new-painkillers-could-replace-opioids.html
There was also dedicated interest from pain advocacy groups and pain practitioners, e.g. a 1p-full-page coverage in the August issue of "*PainMedicineNews*", the most widely read forum on Pain Medicine in the US and in English-speaking countries.
http://www.painmedicinenews.com/Science-Technology/Article/08-16/Study-'Novel-Compounds'-Coinhibit-Two-TRP-Channels-Related-to-Inflammation-and-Pain/37464
- Another interview with NPR is scheduled, indicating sustained interest of the general public in this topic.

Participants
Co-PIs Wolfgang Liedtke and Russell Hall$_{III}$, Co-I Jennifer Zhang;
Research Scientist Patrick Kanju, Michele Yeo, Whasil Lee, and Research Technician Yingai Jin

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Small molecule dual-inhibitors of TRPV4 and TRPA1 for attenuation of inflammation and pain

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TRPV4 ion channels represent osmo-mechano-TRP channels with pleiotropic function and wide-spread expression. One of the critical functions of TRPV4 in this spectrum is its involvement in pain and inflammation. However, few small-molecule inhibitors of TRPV4 are available. Here we developed TRPV4-inhibitory molecules based on modifications of a known TRPV4-selective tool-compound, GSK205. We not only increased TRPV4-inhibitory potency, but surprisingly also generated two compounds that potently co-inhibit TRPA1, known to function as chemical sensor of noxious and irritant signaling. We demonstrate TRPV4 inhibition by these compounds in primary cells with known TRPV4 expression - articular chondrocytes and astrocytes. Importantly, our novel compounds attenuate pain behavior in a trigeminal irritant pain model that is known to rely on TRPV4 and TRPA1. Furthermore, our novel dual-channel blocker inhibited inflammation and pain-associated behavior in a model of acute pancreatitis – known to also rely on TRPV4 and TRPA1. Our results illustrate proof of a novel concept inherent in our prototype compounds of a drug that targets two functionally-related TRP channels, and thus can be used to combat isoforms of pain and inflammation *in-vivo* that involve more than one TRP channel. This approach could provide a novel paradigm for treating other relevant health conditions.

Transient receptor potential Vanilloid 4 (TRPV4) ion channels were initially discovered as osmotically-activated channels[1,2]. Discussing the channel's possible role as mechanosensor, and its expression in sensory neurons in the trigeminal and dorsal root ganglion[1,3,4], led to postulation and eventual experimental validation of a possible function in pain sensing and signaling[1,3–5]. This medically-relevant role was corroborated over time[6–15], as was the mechano-sensory role of TRPV4[11,16–20]. The pro-nociceptive prostanoid PGE2, activation of PAR-2 signaling, inflammation and nerve injury were found to augment TRPV4-mediated pain signaling in various systems[5,6,9,12,21,22], including a novel model of temporo-mandibular joint (TMJ) pain[14]. In a shift of paradigm, TRPV4 was found to function as a relevant sensing molecule in epidermal keratinocytes for UVB overexposure[15]. UVB-exposed keratinocytes, depending on their TRPV4 expression and signaling, were functioning as organismal pain generators, supported by the finding that deletion of *Trpv4* exclusively in these cells sufficed to greatly attenuate the organismal pain response. TRPV4 was also found to play a role in visceral pain, e.g. of the colon and pancreas[7,8,18,23–25], the latter two conditions also co-involving TRPA1[8,24,26–28]. The co-involvement of TRPV4 and TRPA1 was also noted in our TMJ model[14], as well as in formalin-mediated irritant pain of the trigeminal territory, which serves as a generic model of cranio-facial pain[13].

Importantly, blocking TRPV4 with selective inhibitors shows similar results as those obtained with genetic knockouts[13,14,25,29–34], particular in models of TMJ pain or formalin-induced trigeminal formalin pain[13,14]. These findings suggest that TRPV4 could serve as a critical pain target, thus incentivizing the development of more potent and selective small-molecule inhibitors as new clinically-relevant therapeutic drugs. This direction has

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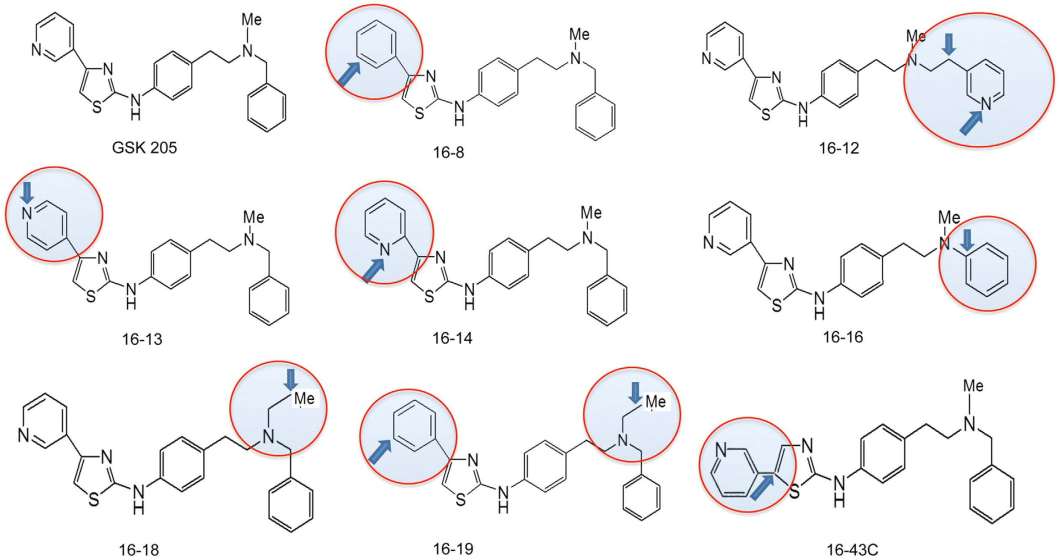

Figure 1. Modifications of tool compound GSK205 for improved targeting of TRPV4. The synthesized compounds differed in the highlighted part of the molecule, changed residue indicated with arrow. Compound 16-19 compound was synthesized to incorporate two modifications from two compounds, 16-8 and 16-18, found most potent in anti-TRPV4 screening assays (see Fig. 2).

advantageous features because genetic approaches are currently limited to experimental conditions and TRPV4 inhibitors are not yet clinically available

The goal of this study was to develop TRPV4 inhibitors with increased potency over a previously used tool compound, GSK205[32–34]. Our results indicate that we have successfully developed compounds with significantly increased TRPV4-inhibitory potency as compared to the tool compound. Interestingly, our approach led to the development of two novel inhibitor molecules that simultaneously target TRPV4 and TRPA1, a potentially advantageous property that we successfully applied in two exemplary *in-vivo* preclinical models of pain, irritation and inflammation.

Results

Chemical synthesis of GSK205 derivatives and assessment of their TRPV4-inhibitory potency in cell-based assays.

We modified compound GSK205 by generating 7 primary modifications, as shown in Fig. 1. One additional compound (16-19) that had the combined respective modifications of the two most potent compounds, as defined in primary screens, was also synthesized. We assessed TRPV4-inhibitory potency of these synthetic compounds in a Ca^{++} imaging assay in neuronal 2a (N2a) permanent tissue culture cells with directed expression of mammalian (rat) TRPV4. TRPV4 channels were stimulated with a selective activator compound, GSK1016790A (GSK101), used at 5 nM. For first round assessment, all TRPV4-inhibitory compounds were used at 5 μM (Fig. 2A). Compound 16-43C did not inhibit Ca^{++} influx, and its effect was similar to vehicle control. All other compounds inhibited TRPV4-mediated Ca^{++} influx, with compounds 16-8 and 16-18 emerging as the two most potent. Compound 16-19 which incorporated the modifications of both 16-8 and 16-18, was also effective in inhibiting TRPV4-mediated currents. However, we did not find a significant difference between compound 16-19 and 16-8, both of which virtually eliminated Ca^{++} influx.

We then conducted more detailed dose-response assessments for compounds 16-8, 16-18 and 16-19, which yielded an IC_{50} of 0.45, 0.81 and 0.59 μM, respectively, vs. an IC_{50} of 4.19 μM for parental compound GSK205. These findings represent an increased potency of the GSK205-derivative compounds by approximately 10-fold for 16-8, 8-fold for 16-19 and 5-fold for 16-18. Surprisingly, compound 16-19 was not significantly more potent than 16-8, whereas 16-8 was more potent than 16-18. Based on these results, we tested 16-8 and 16-19 vs GSK205 (as a control) in patch-clamp studies (Fig. 3). Our results indicate significantly increased potency of compounds 16-8 and 16-19 as compared to the parental molecule, GSK205 (all applied at 5 μM) in attenuating TRPV4-mediated currents.

We next decided to assess potency of the most potent compound 16-8 vs. parental compound GKS205 in two types of primary cells that are known to express TRPV4 and in which TRPV4 function has been demonstrated in a relevant biological context. We examined articular chondrocytes, which have prominent TRPV4 expression, where TRPV4 serves as the mechanotransducer of physiologic mechanical loads to regulate the cells' anabolic response, and thus tissue homeostasis, in cartilage[19]. In addition, we studied brain astrocytes, where TRPV4 expression and relevant function has been previously demonstrated in regulating astrocyte cellular edema, in the coupling of neuronal activity to cerebral blood flow, and in mediating CNS traumatic injury[35–37]. Fulfilling our main objective, in both cell types we observed significantly increased potency of compound 16-8 as compared to the parental molecule, GSK205 (Fig. 4). Evaluation of the inhibitory potency of GSK205 derivatives in these primary cells, which express functional and biologically-relevant TRPV4 without

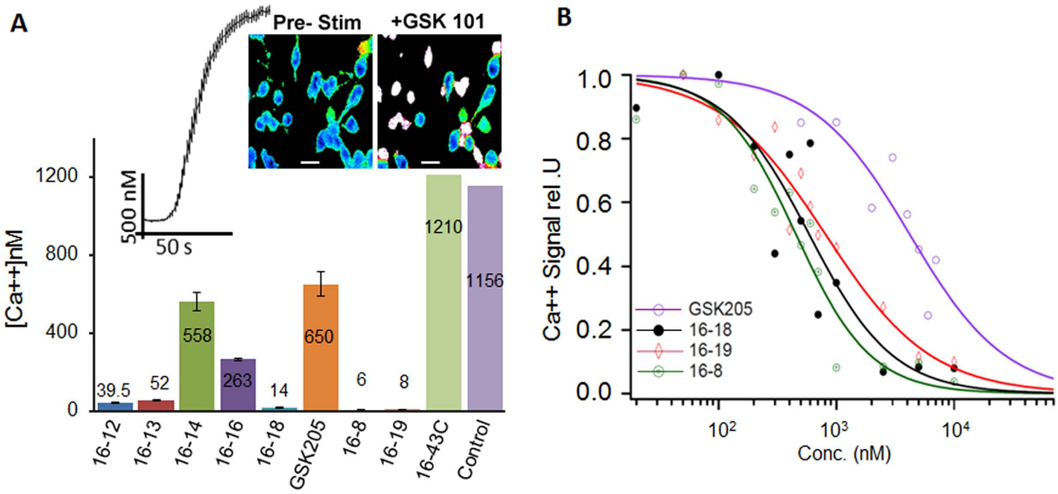

Figure 2. Assessment of 16-… compounds in N2a cells with directed expression of TRPV4. (**A**) Ca^{++} imaging screening of all compounds in N2A cells with directed expression of TRPV4 (rat). The cells were stimulated with TRPV4-selective activator compound, GSK101 (5 nM) in the presence of 5 µM of the respective inhibitor. The number on each bar corresponds to average peak ΔCa^{++} concentrations in ≈100 cells. Inset: micrographs of pseudo-colored cells before and after activation with 5nM GSK101, in addition note the corresponding time course of the averaged Ca^{++} signal (fura-2 Ca^{++} imaging). Except for compound 16-43C, the difference to vehicle control reach the level of statistical significance $p < 0.01$ (one-way ANOVA). **(B)** Dose-response of the most potent, "winner" compounds in TRPV4-expressing N2a cells. The IC_{50} were; 0.45 ± 0.05 µM (16-8), 0.59 ± 0.12 µM (16-18), 0.81 ± 0.1 µM (16-19), 4.19 ± 0.71 µM (GSK205). Plot generated from averaged peak ΔCa^{++} concentration of ≥75 cells per data-point.

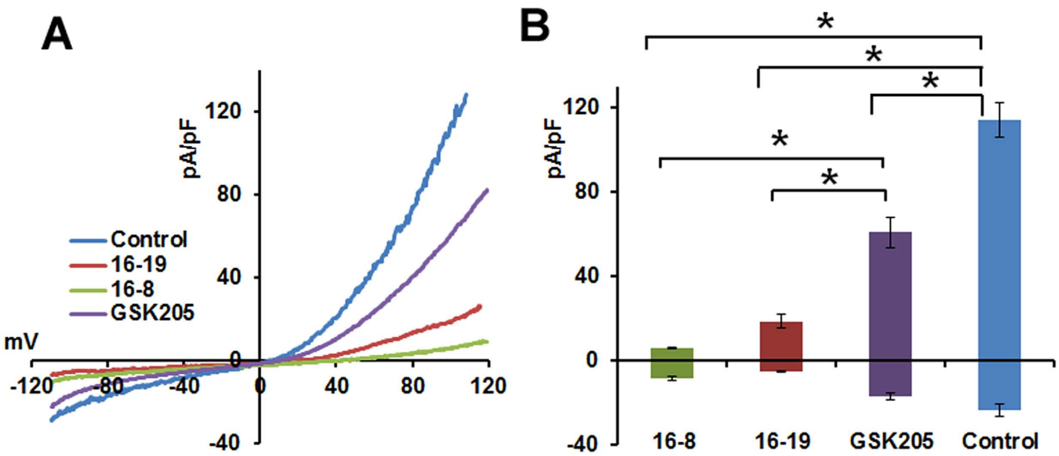

Figure 3. TRPV4 channel inhibition by compounds 16-8 and 16-19 – patch-clamp e-phys. (A) Current-voltage relationship of TRPV4-mediated currents after activation with 5 nM GSK101. Recordings were performed in TRPV4-GFP+ N2a cells. The representative traces represent an average of ≈12 sweeps. In all experiments, cells were pre-incubated with the respective compound (5 µM) for 5 minutes. **(B)** Average current densities at −100mV/+100 mV were significantly diminished by inhibitors (*$P < 0.05$; one-way ANOVA; n ≥ 5 cells/group).

directed over-expression of the channel (Fig. 4), directly corroborate the findings of more basic studies using heterologously TRPV4-overexpressing immortalized cell lines (Fig. 2), strongly supporting our conclusions on the increased potency of the newly derived compounds. Taken together, we identified compound 16-8 as a TRPV4-inhibitory compound with sub-micromolar potency in heterologous systems, with approximately a 10-fold increase in potency as compared to its parental molecule, GSK205. Moreover, 16-8 proved more effective in TRPV4-expressing primary skeletal and CNS-derived cells. However, the rational modification to compound 16-19, intended to further enhance potency, did not yield the intended effect.

Before testing these compounds in relevant *in-vivo* animal models, we next tested their cellular toxicity as well as the specificity of these compounds against other selected TRP ion channels.

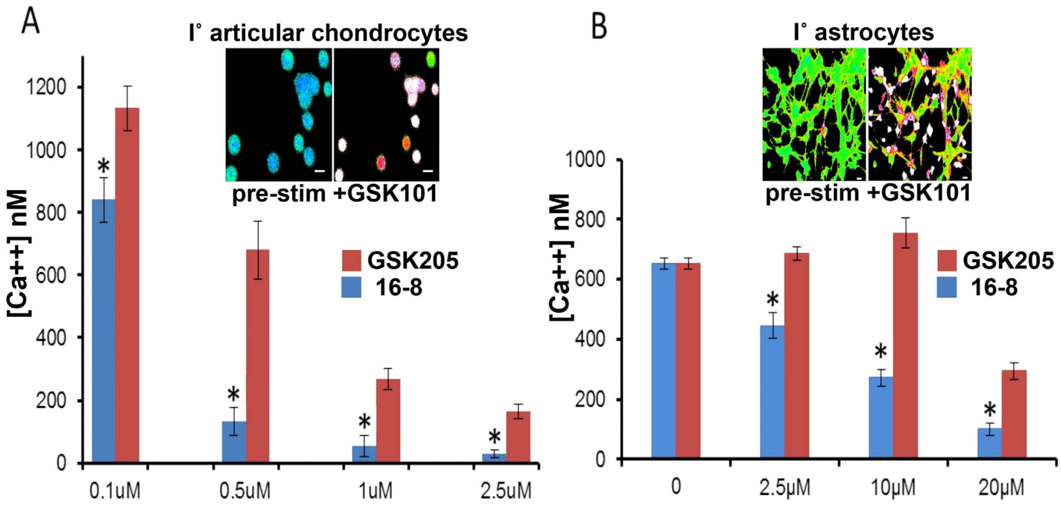

Figure 4. Compound 16-8 inhibits TRPV4 in I° cells more potently than GSK205. **(A)** I° articular chondrocytes (pig); dose-response comparison between the most potent compound, 16-8, and GSK205 in response to stimulation with 5 nM GSK101. Inset: Chondrocytes responding to activation with GSK101, fura-2 Ca^{++} imaging; right-hand image taken at 5 sec after GSK101 application. 16-8 was significantly more potent than GSK205 (mean ± SEM, n = 6 independent expts, n ≥ 25 cells/expt; *$p < 0.05$, t-test). Ordinate shows average peak ΔCa^{++} concentrations. **(B)** I° astrocytes (rat); dose-response comparison between 16-8 and GSK205 in response to 5 nM GSK101. Inset: Astrocytes responding to activation with GSK101; right-hand image taken at 5 sec after GSK101 application (mean ± SEM, n = 5 independent expts, n ≥ 200 cells/expt; *$p < 0.05$, t-test). Ordinate shows average peak ΔCa^{++} concentrations.

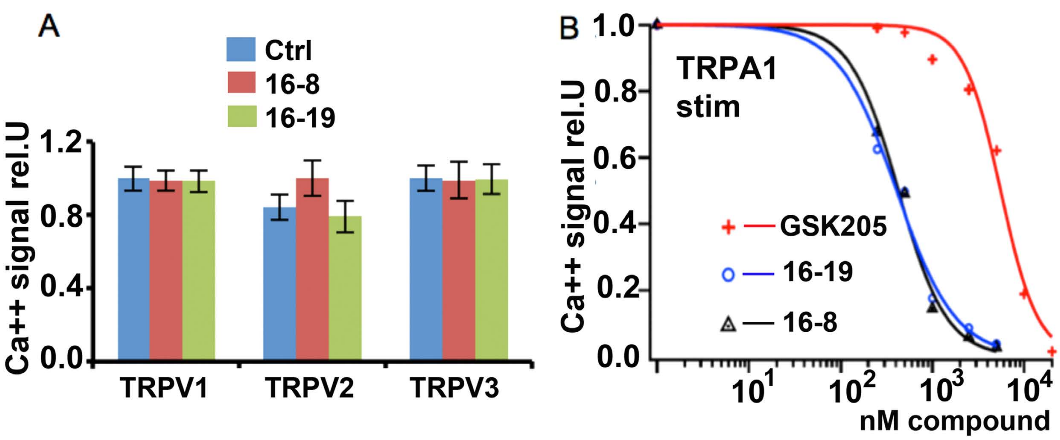

Figure 5. Compounds 16-8 and 16-19 also potently inhibit TRPA1, not TRPV1-3. **(A)** Specificity vs TRPV1-3. Both 16-8 and 16-19 (5 μM each) compounds did not inhibit TRPV1, −2 or −3 channels (all mouse isoforms), directed over-expression in N2a cells and subsequent Ca^{++} imaging. Mean±SEM is shown, ≥100 cells per condition. **(B)** Dose-dependent inhibition of TRPA1 (mouse, directed expression in N2a cells) by GSK 205, 16-8 and 16-19, activation with 100 μM mustard oil, resulting in IC_{50} of 5.56 ± 0.4 μM (GSK205), 0.41 ± 0.37 μM (16-19), 0.43 ± 0.3 μM (16-8). Plot generated from averaged peak ΔCa^{++} concentration of ≥75 cells per data-point.

Novel TRPV4 inhibitors are selective, with benign toxicity profile, yet display potent inhibition of TRPA1. In heterologously transfected permanent N2a cells, we did not observe inhibitory potency of compounds 16-8 or 16-19 toward TRPV1, TRPV2 and TRPV3 (Fig. 5A). However, we made the unexpected discovery of sub-micromolar inhibitory potency vs TRPA1 for compounds 16-8 and 16-19, micromolar potency for GSK205, and, remarkably, no significant activity for compound 16-18 (Fig. 5B). We recorded IC_{50} of 0.41, 0.43, 5.56 μM and >25 μM for compounds 16-8, 16-19, GSK205 and 16-18, respectively.

In terms of cellular toxicity, we found first evidence of toxicity using a sensitive cell viability assay over a time-course of 2 days, at 20 μM, and more pronounced effects at 40 μM (Fig. 6).

Taken together, our results indicate that compounds 16-8 and 16-19 also inhibit TRPA1 at sub-micromolar potency, and their cellular toxicity vs their inhibitory potency against TRPV4/TRPA1 ranges at a factor of 50–100.

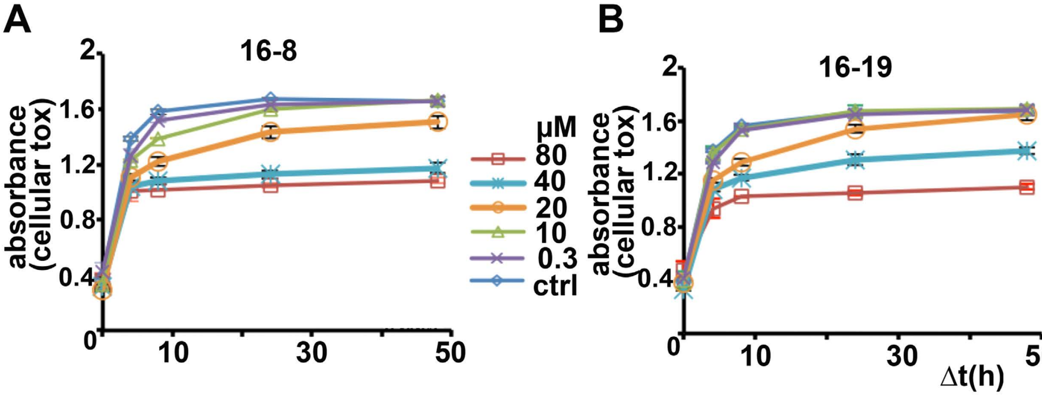

Figure 6. Cellular toxicity studies of compounds 16-8 and 16-19. N2a cells were subjected to increasing concentrations of compounds 16-8 and 16-19, resulting cell viability was analyzed for the next 48 h. **(A)** Time course of cell viability in the presence of various concentrations of 16-8. Note clear reduction at 40 and 80 μM. **(B)** As in (**A**), for compound 16-19, with similar outcome. Representative result of 2 independent experiments.

Assessment of specificity of 16-8, 16-18 and 16-19 against a wider spectrum of receptors and ion channels will be the subject of dedicated future studies directed toward translation of these compounds to the clinic.

We next evaluated these compounds to an *in-vivo* model of irritant pain known to rely on both, TRPV4 and TRPA1.

TRPV4/TRPA1 dual-inhibitors are effective in containing trigeminal irritant pain. We have previously described TRPV4 as a cellular receptor for formalin[13], and demonstrated its involvement in formalin irritant-evoked pain behavior, with a focus on trigeminally-mediated irritant pain behavior. In this recent study, we also demonstrate the co-contribution of TRPV4 together with TRPA1 in trigeminal formalin-evoked pain. We also showed the effective attenuation of trigeminal formalin-evoked pain behavior using GSK205 in a dose-dependent manner. Moreover, we found irritant pain behavior in response to selective activation of TRPV4 in the trigeminal territory, which was blocked by GSK205, and the absence of such an effect in *Trpv4*$^{-/-}$ pan-null mice.

With this pertinent background as a rationale, we applied TRPV4/TRPA1 dual-inhibitory compounds 16-8 and 16-19 systemically, using GSK205 as control, at 10 mg/kg dosage. We prioritized the dual-inhibitors over testing of compound 16-18 (TRPV4-only inhibitor) because (i) the trigeminal formalin pain model relies on both TRPV4 and TRPA1, (ii) we intended to develop TRPV4/TRPA1 dual-inhibitory molecules toward translational use in the first place. None of the compounds were effective at significantly diminishing pain behavior in the early phase after formalin whisker-pad injection, which represents an acute chemical tissue injury pain. In the delayed phase, which is understood as neurally-mediated pain indicative of early maladaptive neural plasticity, there was a significant attenuation of formalin-evoked pain behavior in response to compound 16-8 and 16-19, with compound 16-8 diminishing pain behavior at a remarkable >50%[13], and compound 16-19 also showing a robust effect (Fig. 7A,B). Of note, at 10 mg/kg systemic application, there was no significant effect of GSK205, which was effective previously in a dose-dependent manner when applied by intradermal injection[13]. Thus, compounds 16-8 and 16-19, upon systemic application, effectively attenuate the late, neurally-mediated phase of trigeminal formalin pain, and these compounds are more potent *in-vivo* than their parental compound, GSK205.

In view of these *in-vivo* findings, taken together with the results from heterologous cellular expression systems that indicate an additional TRPA1-inhibitory effect of compounds 16-8 and 16-19, we decided to assess effectiveness of these compounds in a setting of genetically-encoded absence of *Trpv4* (*Trpv4*$^{-/-}$ mouse), in order to better define their TRPA1-inhibitory potency *in-vivo*. We observed significant residual irritant-pain behavior in all phases of the formalin model in *Trpv4*$^{-/-}$ mice, consistent with our previous report[13] (Fig. 7C,D). Immediate-phase pain behavior was virtually eliminated with compounds 16-8 and 16-19, both applied again at 10 mg/kg body-weight. Late-phase pain behavior was strikingly reduced when applying compound 16-8, and still significantly reduced vs vehicle-treated *Trpv4*$^{-/-}$ when applying compound 16-19, although not as potently as 16-8. A reference TRPA1-inhibitory compound, A967079, was used at 25 mg/kg body weight as a positive control to inhibit TRPA1, based on a previous report[38]. Reduction of pain behavior in *Trpv4*$^{-/-}$ mice was striking, more than 50% in the late neural phase. We noted equal potency of compound 16-8 at 10 mg/kg body weight vs. reference TRPA1-inhibitory compound A967079 at 25 mg/kg body weight, both reducing formalin-evoked trigeminal pain behavior to similarly robust degree (Fig. 7C,D). We conclude that compound 16-8 also functions as a potent TRPA1-inhibitor in an *in-vivo* irritant pain model specifically designed to assess the contribution of TRPA1 to trigeminal irritant pain, and at least as potent as an established reference TRPA1-antagonistic compound.

Potent TRPV4/TRPA1 dual-inhibitor, 16-8, is effective at controlling inflammation and pain in acute pancreatitis. These findings define compound 16-8 as a potent TRPV4/TRPA1 dual-inhibitor molecule, based on cell-based and live-animal results. We therefore decided to test it in a more specific preclinical pain model that relies on both, TRPV4 and TRPA1, in order to establish proof-of-principle that a dual-inhibitor can effectively treat pain and inflammation in pancreatitis. We used a pancreatitis model because it provides high

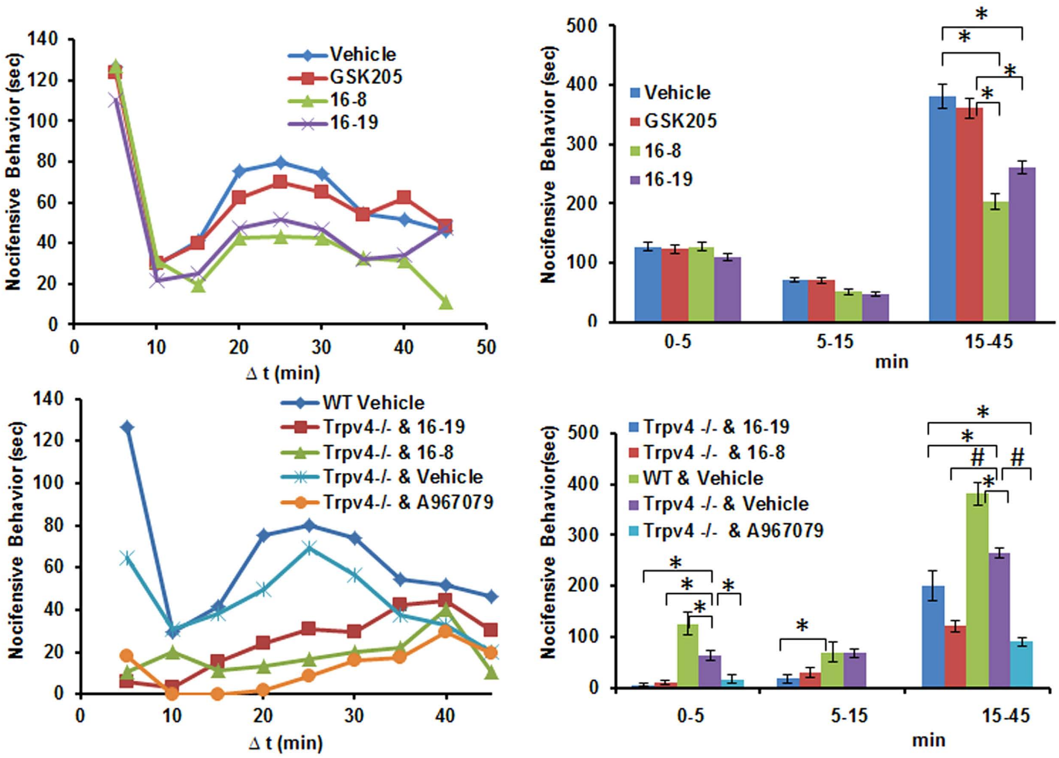

Figure 7. 16-8 and 16-19 effectively attenuate formalin-evoked trigeminal irritant pain. **(A)** Time-course of nocifensive behavior in WT mice following whisker-pad injection of 4% formalin. The mice were pre-injected (i.p., 10 mg/kg; 15 min before formalin) with GSK205, 16-8 or 16-19. Note effective reduction of nocifensive behavior in the late "neural" phase by compounds 16-8, 16-19, not by GSK205. **(B)** Cumulative response binned into 3 phases: acute phase (0–5 min), interphase (5–15 min), and late "neural" phase (15–45 min). Note significant reduction of nocifensive behavior in the late phase by 16-8, 16-19, not GSK205 (*$P < 0.01$ vs vehicle and GSK205, one-way ANOVA). **(C)** As in (**A**), but also including *Trpv4*$^{-/-}$ mice. Compounds were applied i.p. 15 min before formalin challenge, at 10 mg/kg except established TRPA1 blocker, A967079 (25 mg/kg). Previously-established attenuated nocifensive behavior in early and late phase in *Trpv4*$^{-/-}$ mice was recapped, which was reduced further by TRPA1 blocker, A967079. **(D)** As in (**B**), plus inclusion of *Trpv4*$^{-/-}$ mice. Robust effects of TRPA1-blocker, A967079, were mimicked equi-potently by 16-8 and 16-19 for early phase, and by 16-8 for late phase, partially by 16-19 for late phase. (**A,C**) show averaged behavioral metrics per time-point, bars in (**B,D**) represent mean ± SEM; for (D) $^{*}P < 0.05$; $^{\#}P < 0.005$, one-way ANOVA; for all panels n = 5–8 mice/group.

translation potential due to unmet clinical need for new effective treatments in this condition[39], as well as the known role of both TRPV4 and TRPA1 in pancreatitis pain and inflammation[8,25,40].

Pancreatitis was induced with caerulein, a well-characterized model for acute pancreatitis[41]. Animals were treated with 10 mg/kg bw 16-8 by intraperitoneal injection, 30 min before induction of inflammation. We found significant attenuation of inflammatory parameters, namely edema, which was virtually eliminated in 16-8 treated animals. Furthermore, serum amylase, a marker of inflammatory injury of the pancreas, was significantly reduced by 16-8 treatment, as was myelo-peroxidase content of the pancreas, as a marker of inflammatory cell infiltration of the pancreas. The histopathological score for pancreas inflammation was also significantly reduced (Fig. 8A–E). Of note, pain behavior, similar to the effect of 16-8 on pancreas edema, was virtually eliminated upon treatment with compound 16-8. Thus, compound 16-8 was found to be highly effective in attenuating pain and inflammation of acute chemically-evoked pancreatitis.

Benign preliminary toxicity and pharmacokinetics of novel TPRV4/TRPA1 inhibitors. Promising properties of potent 16-… compounds prompted us to attempt to define their initial preliminary features in terms of *in-vivo* toxicity and pharmacokinetics, which will be followed by more extensive and in-depth investigations in future studies. For this, we chose compound 16-8 as the all-around most potent dual TRPV4/TRPA1 inhibitor, and also compound 16-19 with its potentially increased lipophilicity (Suppl Table 1). We measured compound concentration in several organs and plasma, and detected micromolar/submicromolar concentrations in liver and kidney, less than 100 nM concentrations in heart, brain, brainstem, trigeminal ganglion and skin. Of note, compounds were virtually undetectable in plasma (Suppl Fig. 1A). We detected higher concentrations of 16-19 in non-nervous tissue, especially liver and kidney, a pattern perhaps related to compound 16-19's increased lipophilicity. Based on this finding, we next examined 6 and 24 h time-points and detected 10-20 fold higher concentrations of 16-19 at the 6 h time-point, compared to the 1 h time-point, indicative of compound sequestration

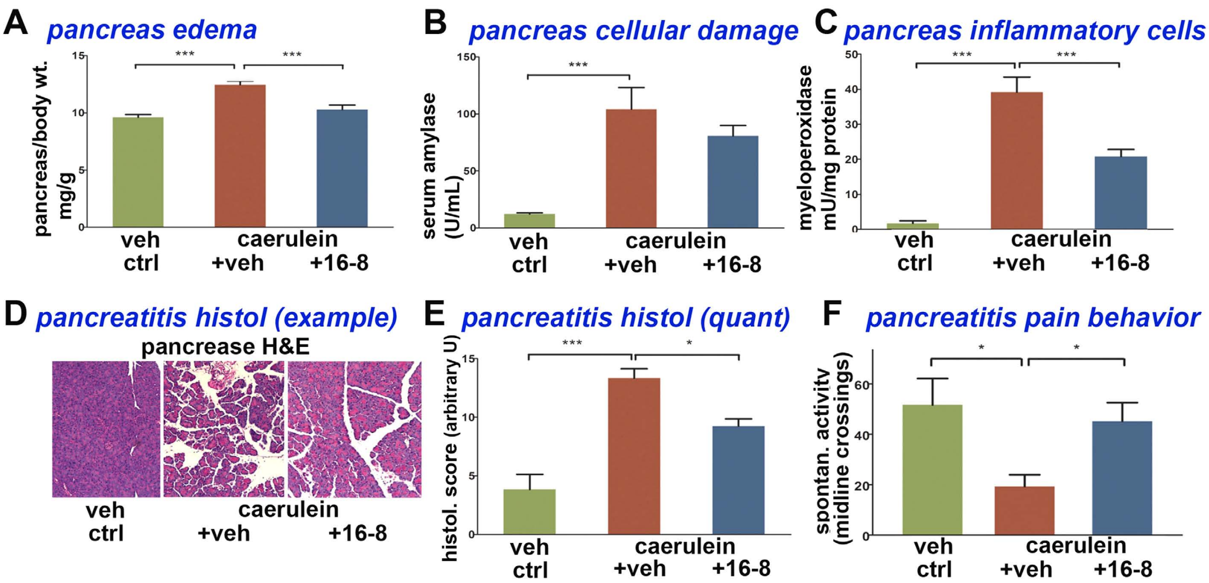

Figure 8. Compound 16-8 attenuates acute pancreatitis and improves pain behavior. (A) Caerulein-evoked acute pancreatitis causes pancreatic edema, which is eliminated by compound 16-8 (10 mg/kg, applied at 30 min before first exposure to caerulein). **(B)** Caerulein-evoked acute pancreatitis strongly elevates cellular toxicity marker amylase in serum. Amylase is reduced, but not significantly, in 16-8 treated animals. **(C)** caerulein-evoked acute pancreatitis causes elevated myelo-peroxidase (MPO) activity in serum, a marker for infiltration of inflammatory cells into the pancreas. MPO activity is significantly reduced in 16-8 treated mice. **(D)** caerulein-evoked acute pancreatitis can be readily demonstrated histologically, exemplified in the micrograph panels shown. Note increased pancreas inflammation in the middle-panel vs non-inflamed pancreas in vehicle-control challenged mice, and its attenuation by treatment with compound 16-8. **(E)** Bar diagram shows quantitation of inflammatory histologic parameters as shown in (**D**). Note significant increase of inflammation-index in caerulein acute pancreatitis mice, and its significant reduction upon treatment with compound 16-8. **(F)** Caerulein-evoked acute pancreatitis causes pain behavior, significantly reduced by compound 16-8. Note greatly reduced activity over the 6 h test period in caerulein-induced acute pancreatitis. This nocifensive behavior is greatly improved in response to systemic application of compound 16-8. Results are expressed as mean ± SEM; n = 6 mice/group; *$P < 0.05$ (one-way ANOVA).

into solid organs (Suppl Fig. 1B–D). Values at the 24 h time-point were lower than at the 6 h but higher than at the 1 h time-point, indicating ongoing protracted compound clearance. We next confirmed that low/non-detectable concentrations in plasma were not caused by compound denaturation/degradation in plasma, as indicated in Suppl Fig. 1E, which shows no loss of detectable compound after 4 h incubation in plasma at 37 °C, conducted using compound 16-19.

We next performed basic preliminary *in-vivo* toxicity studies for compounds 16-8 and 16-19, both at 10 mg/kg bw, which was the effective concentration in both *in-vivo* pain models. We did not detect first evidence of cardiac, hepatic and renal toxicity, when comparing compounds 16-8 and 16-19 with vehicle (Suppl Fig. 2). For cardiac assessment, we did not detect differences and changes in heart rate over 1 h, conducted by EKG at the 6 h time-point. Serum creatinin as marker of renal function and alanine-amino-transferase (ALT) as marker of hepato-cellular integrity were not significantly elevated in animals treated with compounds 16-8 or 16-19. Thus, initial evidence for potent 16-… compounds highlights their acceptable preliminary pharmacokinetics properties as well as lack of gross systemic toxicity. Future studies will be necessary for more detailed assessment of the pharmacokinetics and toxicity of these compounds.

Discussion

Here we describe novel compounds that simultaneously inhibit both TRPV4 and TRPA1 ion channels. Both targets were inhibited by the novel "dual-inhibitors" at sub-micromolar potency in heterologous cellular channel activation assays. Furthermore, these compounds showed potent activity against TRPV4 in primary cells with native TRPV4 expression, and were more potent than their related parent-compound. The most potent compound identified here, compound 16-8, also showed a favorable activity profile in two pain-inflammation models, one of them a general irritant-pain model in the trigeminal system, the trigeminal formalin model, the other a visceral pain and inflammation assay with specificity for the pancreas, the caerulein-induced acute pancreatitis model. Of note, both *in-vivo* pain models have been shown previously to rely on co-contribution of TRPV4 and TRPA1. In this regard, several other relevant medical conditions, discussed in more detail below, also rely on TRPV4/TRPA1 and represent important unmet clinical needs that need to be addressed by translational-medical approaches. Therefore a potent "dual-inhibitor" for a specific combination of TRP ion channels, such as TRPV4 and TRPA1, could be highly beneficial in these indications.

More specifically, primary chondrocytes represent a cellular model for joint disease with involvement of TRPV4 in cartilage maintenance as well as arthritis/osteoarthritis. Recent evidence also suggests a potential role for TRPA1 in mediating joint pain in osteoarthritis[42]. In addition, TRPV4-expressing astrocytes are involved in many neurologic and psychiatric diseases such as pain, epilepsy, multiple sclerosis and other CNS autoimmune conditions, stroke, traumatic brain/spinal cord injury, brain edema, CNS infections, and more[35–37]. TRPA1 expression in astrocytes has also been reported[43]. Therefore, novel dual TRPV4/TRPA1 inhibitors might be suitable compounds for treatment of a number of diseases, from a spectrum of disorders affecting the nervous system as well as degenerative or inflammatory musculoskeletal conditions. For both types of disorders, we view compartmentalized application of compounds, i.e. intra-thecal or intra-articular delivery, as feasible future routes of delivery, in order to affect target cells more directly without affecting - TRPV4 and TRPA1 systemically.

In terms of *in-vivo* pain models, the trigeminal formalin model is rather a general model, not a direct pre-clinical translational-medical model. However, formalin models have a very robust track record in the identification of efficacious new compounds against pain[44]. Our findings with compound 16-8 in the trigeminal formalin model can be interpreted along these lines, especially for trigeminal pain including headaches[45,46]. In the context of these most prevalent neurological diseases, involvement of TRPV4 and TRPA1 has been reported previously in preclinical models, underscoring the case for their involvement with some compelling preclinical insights for both channels[47–50].

Pancreatitis in mice represents a more specific preclinical visceral inflammatory pain model, helpful to elucidate pathophysiology of this specific pain in order to better address a significant unmet medical need. In the current study pain and edema were strongly reduced by compound 16-8, the compound identified as showing the most advantageous features when tested in the trigeminal formalin pain model vs another high-potency dual-inhibitor, 16-19, and vs parent molecule, GSK205. This is in keeping with previous studies which demonstrated that both TRPA1 and TRPV4 contributed to pancreatic inflammation and pancreatic pain[25]. More precisely TRPA1 was shown to contribute to both pain and inflammation while TRPV4 contributed more selectively toward pain[8]. In our current study, there was significant attenuation of inflammatory parameters, but the reduction was not as robust as for pain and edema. This finding begets two important issues, namely (i) this could be a feature of the 2 targeted TRP channels, that they are more significant for pain than for inflammation (as concluded from experimental evidence in[14]), and (ii) edema of the pancreas, which is readily measurable by imaging techniques in patients, could possibly serve as a bio-marker for pancreatic pain. Beyond its role as biomarker, pancreas edema could sustain pancreas pain. At the pathophysiological level, edema will encompass edematous distension of the inflamed organ causing mechanical pain. This pain, amplified by inflammation of the painful organ, will in turn cause neurogenic inflammation, which will give rise to increased level of edema. This could evolve into a detrimental feed-forward mechanism. The specific effect of compound 16-8 on pancreatitis pain could be due to the compound interfering potently and efficiently with such a feed-forward mechanism as in (ii), by potently inhibiting both channels, as laid out in (i).

Beyond the two pain-inflammation conditions tested, TRPV4/TRPA1 co-involvement appears to play a role in several health-relevant conditions, such as colitis, itch, injury to airway and lungs via the inhalatory route and chronic cough[28,34,51–58]. In addition, interesting recent findings point toward a prominent role for TRPV4 in conditions as diverse as fibrotic disorders, UVB skin injury, and premature birth[15,59,60]. In these conditions, possibly via TRPA1-expressing innervating sensory neurons, a co-contribution by TRPA1 could be an important element. For such cases, 16-… compounds represent attractive candidates for effective treatment, to address the significant underlying unmet clinical need. Except pancreatitis, compound access to relevant target cells could also be readily accomplished by topical, non-systemic delivery via transdermal, transmucosal, inhalatory, intra-articular or intra-thecal formulations.

Our study presented here was strongly geared toward a translational medical agenda, meaning demonstration of effect of TRPV4/TRPA1 dual inhibitors, combined with a first-pass at pharmaco-tox assessment were our priority, rather than in-depth mechanistic studies. In addition, our goal was to demonstrate that modified 16-… compounds were more potent than the parent compound, GSK205. For future studies, in addition to continuation of a translational-medical agenda based on 16-… compounds, e.g. in-depth assessment of 16-… compounds at human isoforms of TRPV4 and TRPA1, effect of 16-… compounds in TRPV4/TRPA1-expressing primary human cells, our current results raise the following important questions/issues, namely a mandate to conduct mechanistic studies that address how TRPV4/TRPA1 dual inhibitors act on their respective target channels, and whether there is perhaps a shared mechanism between TRPV4 and TRPA1 of channel inhibition by potent 16-… compounds. In this context, it will be rewarding to zero in on a potential mechanism as to why compounds 16-8, 16-19 and to minor degree GSK205 are active against TRPA1 whereas compound 16-18 interestingly is not. Furthermore, the question why compound 16-19 fails to show increased potency over 16-8 will be an interesting one to address in future studies.

Materials and Methods

Compound synthesis. Compound synthesis is explicitly described in Supplementary Information (Suppl Fig. 3 and Suppl Tables 2–5).

Animals. 8–12 week old mice were used throughout the experiments. *Trpv4*$^{-/-}$ mice[3] have been outcrossed to WT (C57BL/6J) background and genotyped by PCR[3,15]. Animals were housed in climate-controlled rooms on a 12/12 h light/dark cycle with water and standardized rodent diet that was available ad libitum. All experiments were conducted in compliance and accordance with the guidelines of the NIH and the Institutional Animals' Care and Use Committee (IACUC) of Duke University, and under a valid IACUC protocol of the Duke University IACUC. All animal methods described in this publication were approved by the Duke University IACUC.

Trigeminal formalin irritant behavior – mouse model. Implementation of this model was conducted as described previously[13].

Video-taped nocifensive behavior was assessed by investigators blinded to genotype and treatment.

Acute pancreatitis mouse model. C57BL/6J male mice 8–10 weeks of age were subjected to acute pancreatitis by intraperitoneal injections of supramaximal doses of caerulein (50 μg/kg) every hour for a total of 6 h, as previously described in[61]. Control animals received 25% DMSO-saline solution by intraperitoneal injection every hour for 6 h. Compound 16-8 was dissolved in this vehicle (10 mg/kg) and injected i.p. 30 min prior to the first injection of caerulein. Animals were sacrificed 1 h after the last injection. Blood was collected and pancreatic tissue was promptly isolated, weighed for determination of pancreas wet weight/body weight ratio. Samples of tissues were fixed overnight in 10% neutral-buffered formalin, paraffin embedded and H&E-stained, or pancreatic tissue was quickly frozen and assessed for myeloperoxidase (MPO) activity. Serum amylase, MPO and histologic evaluation were conducted as described previously[61].

Assessment of nocifensive behavior[8]: Mice were housed in individual cages and video-recorded during the entire experiment. Two mice at a time were observed. Linear movement was measured as one event when mice passed through the median plane of the cage. Analysis began immediately after the first caerulein/vehicle injection and continued until the end of the experiment. Results were expressed as the sum of the movement events spanning the 6 h time-period following the first injection.

Cell cultures. N2a cells were used for directed expression of TRP ion channels as described previously[13]. TRPV4-eGFP from rat was used, previously found to respond to stimulation with GSK101 and hypotonicity in similar manner as native, non-fused TRPV4. All other channels were native channels from mouse, eGFP was co-transfected. Stimulation of over-expressed TRPV4 was conducted with GSK101 (5 nM), TRPV1 with capsaicin (10 μM), TRPV2 with hypotonicity (270 mosmol/L), TRPV3 with camphor (100 μM) and TRPA1 with mustard oil (100 μM). eGFP control-transfected N2a cells did not respond to these stimuli. Ca^{++} imaging was performed as described previously[13,14,34].

To visualize dose-response relationships, Hill plots were conducted using the Igor Pro software program, which derived the plots based on the following equation:

$$y = Base + (Max - Base)/\left\{1 + \left[\frac{x\ half}{x}\right]^{rate}\right\} \tag{1}$$

Primary porcine chondrocytes derived from femoral condyles of skeletally mature pigs were cultured and subjected to Ca^{++} imaging as described previously[19,32,62,63].

Astrocyte cultures were conducted following established protocols[64–66]. Astrocytes were prepared from Sprague Dawley rat embryos (E18). Briefly, the isolated cortices were minced, and then incubated with trypsin and DNase. Dissociated cells were suspended in Dulbeccco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Thereafter, cell suspensions were plated in 75 cm^2 tissue culture flasks (10×10^6 cells/flask) which were pre-coated with poly-L-lysine (10 μg/ml). The cells were maintained in a 10%CO_2 incubator at 37 °C. After 10–12 days, the media was removed and adherent cells were trypsinized (0.25%) and plated out onto coverslips for subsequent Ca^{++} imaging[34,67]. >95% of the cells were found to express astrocyte marker, glial fibrillary acidic protein (GFAP)[68].

Cell viability in culture. N2a cells were cultured in 96 well plates for 24–48 h. Cell viability studies relied on metabolic capability monitored with the indicator dye resazurin. Its reduction to resorufin (indicated by color change dark blue to pink) was monitored over time. Changes in absorbance at $\lambda = 570$ nm were recorded using a microplate reader (Molecular Devices). Metabolically active and viable cells shared the ability to reduce resazurin to resorufin whereas dead cells did not. Eight replicate cultures per experimental point were studied.

Assessment of hepatic, renal and cardiac function in mice treated with 16-… compounds. Mice were treated i.p. with compounds 16-8 and 16-19 (10 mg/kg). Hepatic and renal integrity were analyzed by alanine amino-transferase- and creatinine assays (Sigma), both relying on measurement of absorbance at $\lambda = 570$ nm in 96-well micro-titerplates. 8 technical replicates per animal were performed.

For heart rate assessment in mice treated with 16-8 and 16-19, animals were fitted with two electrodes, one to the ear, via clip, one to the rib-cage, using firm adhesive. Heart rate was monitored and analyzed using axoscope and clampfit 9.2 software (Molecular Devices)

Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS). Mice were treated with 10 mg/kg i.p. of the respective inhibitor. Post-euthanasia harvested tissue was frozen in liquid nitrogen and stored at −80 °C for further analysis.

Frozen tissue samples were partially thawed and cut into 1 mm slices, 5–15 mg tissue, 2-fold excess water (mass/vol.), 6-fold excess acetonitrile (16-… compounds) or methanol (GSK205) containing appropriate amount of internal standard, and 2.5 mm zirconia/silica bead (Biospec Products Inc.) were added to 500-μL polypropylene (PP) conical tube, homogenized in a Fast-Prep apparatus (Thermo-Savant) at speed "4" for 20 sec at room temp, and centrifuged at 13,600 g for 5 min at room temp. Depending on the expected concentration range of the measured compound, the supernatant was diluted 1/4–1/20 (in Mobile phase A, see below) and placed in autosampler for LC-MS/MS analysis.

The LC-MS/MS assay for 16-… compounds and GSK205 was developed on an Agilent 1200 series LC system interfaced with Applied Biosystems API 5500QTrap, a hybrid triple quadruple-linear trap MS/MS spectrometer.

Analyst (version 1.6.1) software was used for mass parameters tuning, data acquisition, and quantification. LC column: 3 × 4 mm RP C18 (Phenomenex, AJ0-4287) was operated at 35 °C. Mobile phase A: 0.1% formic acid, 2% acetonitrile, in LC/MS-grade water; mobile phase B: acetonitrile; flow rate: 1 mL/min, 1:1 MS/MS:waste split. Run time was 4 min. Diverter valve was used to send flow to MS/MS only between 1.2 and 2.5 min. The elution gradient was: 0–0.5 min, 1%B; 0.5–1.2 min, 1–95%B; 1.2–1.5 min, 95%B; 1.5–1.6 min, 95–1%B. Autosampler was operated at 4 °C; injection volume was kept at 10–50 μL. Electrospray ionization (ESI) source parameters were: positive ionization mode, curtain gas flow = 30, ionization potential = 5500 V, temperature = 500 °C, nebulizing gas 1 flow = 30, nebulizing gas 2 = 30, declustering potential = 20 V. 16-… compounds and GSK205 were individually infused as 100 nM solutions in 50%A/50%B at 10 μL/min flow rate and parameters optimized to provide maximal ion count for "parent" and collision-produced ("daughter") MS/MS ions. Parent/daughter quantifier [qualifier] ions utilized: GSK205 (401.1/280[370]), 16-8 (400.1/279.1[91.1], 16-16 (387.1/280[105]), 16-18 (415.2/280[370], 16-19 (414.1/279.2[91.1]. Standard (analyte of interest)/internal standard pairs utilized: GSK205/16-16, 16-8/16-16, 16-18/GSK205, 16-19/16-8.

Calibration samples (n = 6) were prepared by adding pure standard of the measured compound to tissue homogenate (tissue + 2-fold excess water, mass/vol) in the appropriate range needed for the particular dosing regime. Organs studied were analyzed alongside the study samples. The following are typical ranges used (the lower value representing also the LLOQ at 80% accuracy limit, all other calibrator levels at 85% accuracy limit): 0.38–6 nM (plasma), 6–100 nM (skin), 6–48 nM (heart), 7.5–120 nM (brain), 19–300 or 1500–24000 nM (liver), 56–900 or 1500–12000 nM (kidney), 500–8000 nM (fat). Peak integration, calibration, and quantification was performed within Analyst software. The response of the peak area standard/int. std. to nominal concentration was linear with r = 0.999 or better.

Patch Clamp Recordings. Heterologously transfected N2a cells were subjected to patch clamp electrophysiological recordings. Briefly, 24 h after transfection cells were prewashed with extracellular fluid (ECF) which contained (in mM) 1 $MgCl_2$, 10 Glucose, 10 HEPES, 145 NaCl and 2 $CaCl_2$ (pH 7.4, 310mOsM). Cells were then incubated with or without TRPV4 inhibitors in ECF for 5 min before whole cell recording. Cover slips were transferred to a recording chamber mounted on the stage of a Leica inverted microscope that was equipped with fluorescent filters. Transfected cells were identified before patching by their green florescent color. Cells were patched with a 2.5–3.0 MΩ glass electrode pulled from borosilicate glass capillaries using pipette puller (Sutter instruments). The intracellular solution contained (mM) 140 CsCl, 10 HEPES, 1 EGTA, 0.3 Na-GTP, 2 Na_2-ATP, and 2 $MgCl_2$ (pH 7.4, 295 mOsm). Whole cell currents were recorded using pclamp 9.2 software and Axopatch 200B amplifier (Molecular Devices). The cells were first clamped at −65 mV before applying a 1 s voltage ramp from −110 mV to +120 mV. The voltage ramp was applied every 2 seconds for 15 to 20 sweeps. Capacitance was monitored throughout the experimental recordings. Reported data was within ±3 pF.

Statistical Analysis. Data are expressed as mean ± SEM. Two-tail *t*-tests or one-way ANOVA followed by Tukey *post-hoc* test were used for group comparisons. $P < 0.05$ indicated statistically significant differences

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Author Contributions
P.K. conducted experiments, analyzed data, wrote paper, conceptual input. Y.C. conducted experiments, analyzed data. W.L. conducted experiments, analyzed data. M.Y. conducted experiments, analyzed data, wrote paper. S.H.L. conducted experiments. J.R. conducted experiments, analyzed data. R.S. conducted experiments, analyzed data. P.F. conducted experiments, analyzed data. D.M.G. conducted experiments, analyzed data. S.A.S. wrote paper, conceptual input. I.S. conducted experiments, conceptual input. R.A.M. wrote paper, conceptual input, analyzed data. R.A.L. wrote paper, conceptual input, analyzed data. F.G. wrote paper, conceptual input, analyzed data. W.B.L. wrote paper, conceptual input, analyzed data.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

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Supplementary File

Title:

Small molecule dual-inhibitors of TRPV4 and TRPA1 for attenuation of inflammation and pain

Authors:

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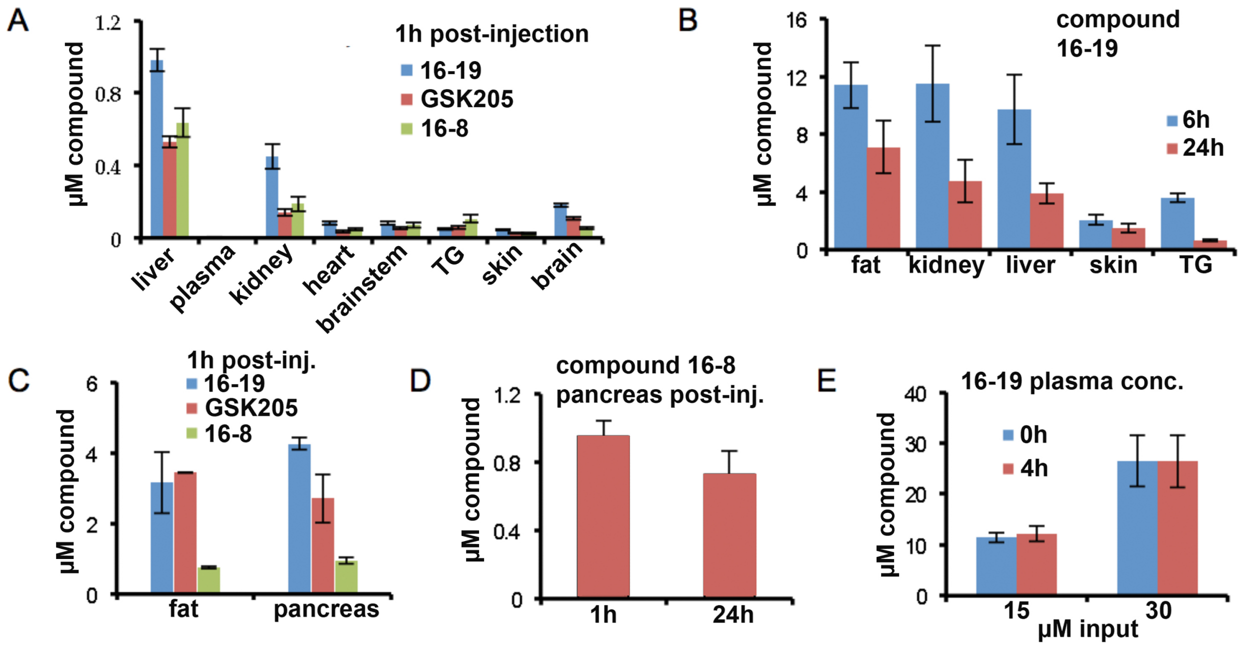
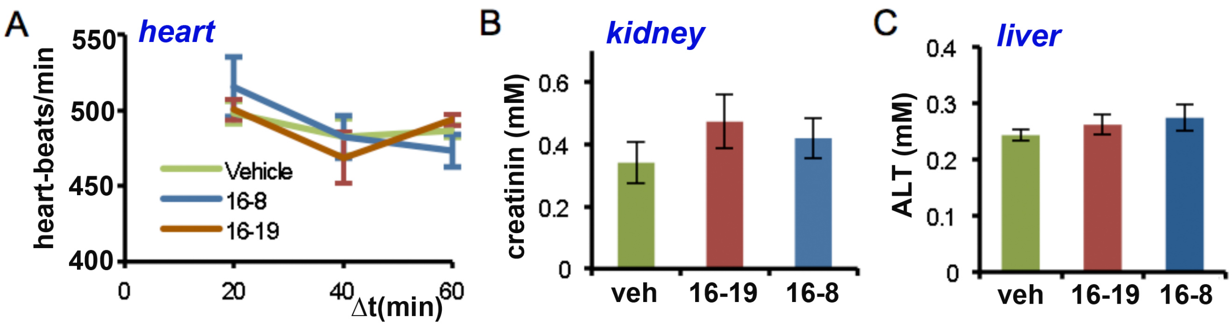


Suppl Fig. 1: *Pharmaco-kinetics/pharmaco-tox of compounds 16-8, 16-19 and GSK205 in-vivo*

(A) concentrations of compounds 16-8, 16-19 and GSK205 (10mg/kg) in several murine tissues/organs 1h post-i.p. injection. **(B)** Compound 16-19 time-course at 6h and 24h in several organs. 16-19 was selected because of its elevated levels at the 1h time-point, and based on the estimate that 16-19 is more lipophilic than 16-8 and GSK205. Note that metrics at 6h are invariably higher than at 24h. All values are appreciably higher than at 1h. **(C)** Concentrations of 16-8, 16-19 and GSK205 in fat and pancreas after one hour. Note lower concentration of 16-8 vs. 16-19 and GSK205, yet above its IC50. **(D)** Concentrations of 16-8 in the pancreas at 1h and 24h time-points. **(E)** Structural stability of compound 16-19 in plasma as suggested by stable concentration after 4h/37˚C. Results are expressed as means±SEM, n=6 mice/experimental group for all expts.



Suppl Fig. 2: *Absence of cardiac, renal and hepatic toxicty of compounds 16-8, 16-19*
(A) Heart rate time-course after i.p. injection (10mg/kg) of compounds. There was no significant difference in heart rates between vehicle and compounds. **(B)** Serum creatinin was not significantly elevated in animals treated with 16-19 and 16-8 vs vehicle control. **(C)** Serum alanin-amino-transferase (ALT) levels were not significantly elevated in animals treated with 16-19 and 16-8 vs vehicle control. n=6 mice/group for all expts.

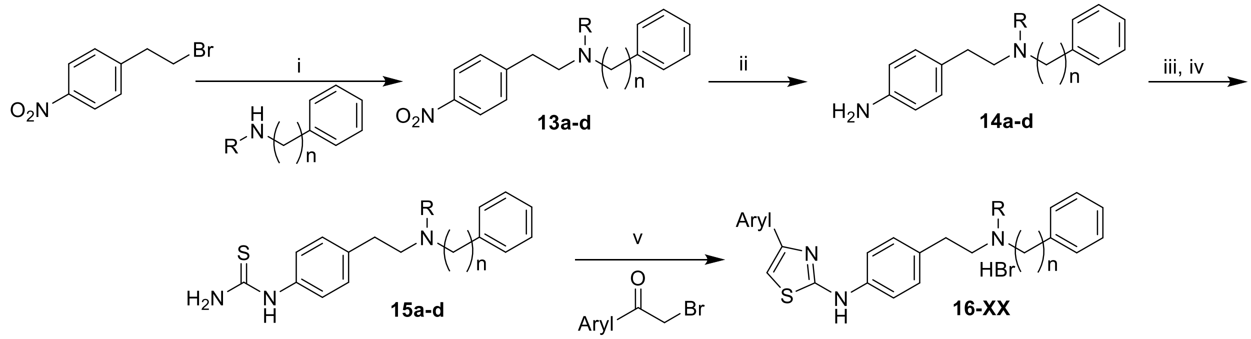
Suppl Table 1

Properties GSK205, 16-8 and 16-19

Compound	**MW**	**PSA**	**c-logP(oct/H_2O)**
GSK205	400	37.53	5.35
16-8	399.6	24.4	6.33
16-19	413.6	24.2	6.58

MW – molecular weight
PSA – polar surface area
c-logP(oct/H_2O) indicates: calculated logP(octanol solubility / H_2O solubility)

Suppl Fig 3 General synthetic scheme for compounds 16-08 to 16-19.



Reagents and conditions: (i) K_2CO_3, CH_3CN. (ii) Zn, MeOH, 12M HCl. (iii) 1,1′-Thiocarbonyldiimidazole. (iv) 7M NH_3 in MeOH. (v) EtOH, reflux

General procedure for the S_N2 displacement of 4-nitrophenethyl bromide: Powdered, oven-dried K_2CO_3 (1.5 eq.) and the amine (1.5 eq.) were added sequentially to a room temperature solution of the bromide (0.33 M) in anhydrous CH_3CN. The reaction mixture was heated to 80 ^oC (oil bath temp) until analysis of the reaction mixture by LCMS indicated complete consumption of the bromide (~6-18h). The mixture was cooled to room temperature and diluted with brine (two volume equivalents). The resulting emulsion was extracted with EtOAc (2 x one volume equivalent). The combined extracts were added to silica gel (mass of silica gel = 2x mass of starting bromide) and the mixture was concentrated to dryness under reduced pressure. Flash column chromatography (RediSepR$_f$ SiO_2, 100% CH_2Cl_2→ 5% MeOH in CH_2Cl_2) gave the product as a brown to amber oil.

Suppl Table 2. Yield of tertiary amines 13a-d

Entry	Compound number	R	n	yield
1	13a	Me	0	17%
2	13b	Me	1	49%
3	13c	Me	2	42%
4	13d	Et	1	15%

General Procedure for the nitro to aniline reduction: A solution of the nitro compound (0.5 M in MeOH) was cooled in an ice-NaCl bath. Zinc dust (4.5 eq.) was added in one portion followed by drop wise addition of 12M HCl (4.5 eq.) over 2-3 minutes. After 1h, the cooling bath was removed and the reaction mixture was allowed to stir over night at room temperature. The following morning, the mixture was cooled in an ice-NaCl bath once again and 30% aqueous NaOH was added drop wise until pH 14 (universal indicating pH paper) was reached. The mixture was diluted with CH_2Cl_2 (five volume equivalents) and stirred for 5 minutes. After this time, insolubles were removed at the vacuum and the filter cake was washed with CH_2Cl_2 (2 x 25 mL). The organic phase of the filtrate was separated, washed with brine (100 mL) and dried ($MgSO_4$). The drying agent was removed by filtration. Silica gel (~5g) was added and the filtrate was concentrated to dryness under reduced pressure. Flash column chromatography (RediSepR$_f$ SiO_2, 100% $CH_2Cl_2 \rightarrow$ 5% MeOH in CH_2Cl_2) gave the product as a clear, amber oil.

Suppl Table 3. Yield of anilines 14a-d

Entry	Compound number	R	n	yield
1	14a	Me	0	75%
2	14b	Me	1	84%
3	14c	Me	2	97%
4	14d	Et	1	85%

General procedure for thiourea formation: A solution of the aniline (0.22 M) in anhydrous CH_2Cl_2 was added drop wise over 2-5 minutes to an ice-NaCl bath cooled solution of 1,1'-thiocarbonyldiimidazole (2 eq., 0.15 M) in anhydrous CH_2Cl_2. After 15 minutes, the cooling bath was removed and the reaction mixture was stirred at room temperature until analysis by TLC (5% MeOH in CH_2Cl_2) indicated complete consumption of the starting aniline. The mixture was cooled once again in an ice bath and 7M NH_3 in MeOH (10.5 eq.) was added drop wise over 2-5 minutes. The bath was removed and the mixture was stirred over night at room temperature. Silica gel (mass of silica gel = 2x mass of starting aniline) was added and the mixture was concentrated to dryness under reduce pressure. Flash column chromatography (RediSepR$_f$ SiO_2, 100% $CH_2Cl_2 \rightarrow$ 10% MeOH in CH_2Cl_2) gave the pure thiourea.

Suppl Table 4. Yield of thioureas 15a-d

Entry	Compound number	R	n	yield
1	15a	Me	0	99%
2	15b	Me	1	96%
3	15c	Me	2	88%
4	15d	Et	1	67%

General procedure for thiazole formation: A mixture of the thiourea (0.1 M) in EtOH and the α-bromoacetophenone derivative (1.1 eq.) was heated to 75 oC (oil bath temperature) until analysis by TLC (5% MeOH in CH2Cl2) indicated complete consumption of the thiourea. Silica gel (mass of silica gel = 2x mass of starting thiourea) was added and the mixture was concentrated to dryness under reduce pressure. Flash column chromatography (RediSepRf SiO2, 100% CH2Cl2 → 10% MeOH in CH2Cl2) gave the pure thiazole hydrobromide**.**

Suppl Table 5. Yield of thiazole hydrobromides 16-08 to 16-19

Entry	Compound number	R	n	aryl	yield
1	16-08	Me	1	phenyl	56%
2	16-12	Me	2	3-pyridyl	82%
3	16-13	Me	1	4-pyridyl	83%
4	16-14	Me	1	2-pyridyl	94%
5	16-16	Me	0	3-pyridyl	98%
6	16-18	Et	1	3-pyridyl	31%
7	16-19	Et	1	phenyl	93%
8	16-43C	Me	1	3-pyridyl	52%

⌘ *Author's Choice*

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Transient Receptor Potential Vanilloid 4 Ion Channel Functions as a Pruriceptor in Epidermal Keratinocytes to Evoke Histaminergic Itch*

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TRPV4 ion channels function in epidermal keratinocytes and in innervating sensory neurons; however, the contribution of the channel in either cell to neurosensory function remains to be elucidated. We recently reported TRPV4 as a critical component of the keratinocyte machinery that responds to ultraviolet B (UVB) and functions critically to convert the keratinocyte into a pain-generator cell after excess UVB exposure. One key mechanism in keratinocytes was increased expression and secretion of endothelin-1, which is also a known pruritogen. Here we address the question of whether TRPV4 in skin keratinocytes functions in itch, as a particular form of "forefront" signaling in non-neural cells. Our results support this novel concept based on attenuated scratching behavior in response to histaminergic (histamine, compound 48/80, endothelin-1), not non-histaminergic (chloroquine) pruritogens in *Trpv4* keratinocyte-specific and inducible knock-out mice. We demonstrate that keratinocytes rely on TRPV4 for calcium influx in response to histaminergic pruritogens. TRPV4 activation in keratinocytes evokes phosphorylation of mitogen-activated protein kinase, ERK, for histaminergic pruritogens. This finding is relevant because we observed robust anti-pruritic effects with topical applications of selective inhibitors for TRPV4 and also for MEK, the kinase upstream of ERK, suggesting that calcium influx via TRPV4 in keratinocytes leads to ERK-phosphorylation, which in turn rapidly converts the keratinocyte into an organismal itch-generator cell. In support of this concept we found that scratching behavior, evoked by direct intradermal activation of TRPV4, was critically dependent on TRPV4 expression in keratinocytes. Thus, TRPV4 functions as a pruriceptor-TRP in skin keratinocytes in histaminergic itch, a novel basic concept with translational-medical relevance.

Itch is a clinical problem that leaves many sufferers insufficiently treated, with >20 million in the United States (1–3). This is also caused by incomplete understanding of its molecular, cellular, and cell-to-cell signaling mechanisms. Neural pathways have been understood as key for itch, whereby specialized primary sensory pruriceptor neurons relay sensory afferent information to itch-transmitting neural pathways, ultimately evoking the sensation of itch (2, 4–8). Exogenous or endogenous pruritogens are thought to act on primary sensory neurons, producing the sensation of itch by activating the pruriceptors expressed by these afferents. Primary pruriceptor neurons may receive modulatory signals from atopic inflammatory cells, such as mast cells, and also from epidermal keratinocytes (7). It was recently elucidated that the atopia cytokine, thymic stromal lymphopoietin (TSLP), was secreted from skin keratinocytes to activate TRPA1 ion channels on primary pruriceptor neurons and induced itch (9). Despite this landmark discovery, mechanisms of how the epidermal keratinocyte specifically functions to evoke itch remain largely unknown, especially mechanistic insights that rely on precise genetic targeting of genes-of-interest only in keratinocytes. In other words, molecular and cell-to-cell signaling mechanisms of forefront pruri-transduction are elusive.

We recently defined a mechanism of how ultraviolet B (UVB)[3] radiation activates TRPV4 ion channels in skin epidermal keratinocytes (10). Their genetically encoded, inducible absence in skin keratinocytes suffices to contain pain and tissue damage evoked by UVB overexposure. In skin keratinocytes, TRPV4 activation by UVB is potentiated by endothelin-1 (ET-1) via endothelin receptors A and B. TRPV4-activation in these cells leads to Ca^{2+} influx, which in turn increases gene expression of ET-1, providing the substrate of a feed-forward mechanism that sustains organismal pain. This is an interesting observation in the context of itch because ET-1 injection into skin is known to cause itch in human subjects and evokes scratching behavior in experimental animals upon intradermal

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[3] The abbreviations used are: UVB, ultraviolet B; TRPV4, transient receptor potential (TRP) vanilloid 4; ET-1, endothelin-1; tam, tamoxifen.

injection (11–16). TRPV4 has been implicated in other forms of pain (10, 17–26). It is a multimodally activated TRPV channel, *e.g.* activated by changes in osmotic pressure, mechanical, UVB, and chemical cues and modified by thermal cues (27–31). Except for the recent elucidation of the role of TRPV4 as ionotropic receptor for UVB in keratinocytes to reprogram these cells into organismal pain generators, its role in pain has been attributed to its expression in primary sensory neurons.

Against this background, especially the finding of TRPV4-dependent secretion of the pruritogen, ET-1, by keratinocytes, we felt that we have raised a timely question, namely whether TRPV4 plays a role in itch, in particular whether TRPV4 in keratinocytes of the epidermis can drive scratching behavior. To address this question we decided to first focus on acute itch and, specifically, as an initial priority, to examine prototypic examples of histaminergic itch, including ET-1-evoked itch, plus chloroquine-caused non-histaminergic itch. In this study we are reporting an exciting new function of TRPV4 in forefront signaling of the integument, namely that TRPV4 in epidermal keratinocytes functions as a pruriceptor-TRP channel in acute histaminergic itch, including itch evoked by ET-1, not in non-histaminergic itch evoked by chloroquine. Direct activation of TRPV4 channels also evokes scratching behavior, which appears completely dependent on TRPV4 expression in keratinocytes, thus underscoring the role of this cell and its expression of TRPV4 in itch. Complementing findings in our *Trpv4* keratinocyte-specific inducible knock-out (*Trpv4* cKO) mice, we demonstrate Ca^{2+} transients in response to histaminergic pruritogens in cultured primary keratinocytes that depend on TRPV4. Ca^{2+} influx via TRPV4 then up-regulates phosphorylation of the mitogen-activated protein kinase ERK in keratinocytes. Consequently, we find topical transdermal treatment with a selective inhibitor of TRPV4 to function efficiently as an anti-pruritogen. Moreover, we observed similar *in vivo* anti-pruritic effects when topically targeting MEK, upstream of ERK, with a selective inhibitor.

Experimental Procedures

Animals—The pan-null phenotype of $Trpv4^{-/-}$ mice relies on excision of the exon encoding transmembrane domains 5–6. Mice were outcrossed to C57BL/6J background and PCR-genotyped (10, 25, 26, 32). Male WT (C57BL/6J) and $Trpv4^{-/-}$, 2–2.5 months of age, were used for all experiments.

Keratinocyte-specific, tamoxifen (tam)-inducible *Trpv4* knockdown mice were used as previously described (10). In brief, the *Trpv4* genomic locus was engineered so that loxP sites surrounded exon 13, which encodes TM5–6. This mutation was propagated in mice that were crossed to K14-CRE-ER^{tam} mice, so that $Trpv4^{lox/lox}$x(K14-CRE-ER^{tam}) mice could be induced by tam (Sigma) administration via oral gavage for five consecutive days at 6 mg/day in 0.3 ml corn oil at age 2–2.5 months of age, plus a 1-time booster 2 weeks after the last application. Control animals received the same volume of corn oil. Efficiency of targeting was verified by quantitative real-time PCR and immunohistochemistry for *Trpv4* expression in skin at gene and protein levels, respectively (10). Both male and female mice were used for *in vivo* scratching behavior as shown in Figs. 1 and 5, and no difference was detected between sexes.

Animals were housed in climate-controlled rooms on a 12/12-h light/dark cycle with water and a standardized rodent diet available *ad libitum*. All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee in compliance with National Institutes of Health guidelines.

Drugs—Histamine, compound 48/80, endothelin-1, chloroquine, and GSK1016790A (GSK101) were purchased from Sigma. GSK2193874 (GSK219) was obtained from Tocris, U0126 was from Selleckchem, and GSK205 was synthesized (26, 33). All were dissolved in sterile normal saline except that GSK101 and GSK205 were dissolved in DMSO (20 mM in stock) and further diluted until use.

Topical Treatment Formulation—Compounds GSK205 and U0126 were kept as DMSO stock, then diluted to 100 μM and 0.1 mg/ml, respectively, in 25% isopropyl alcohol, 15% ethanol, and 60% glycerol when used.

Itch Behavioral Tests—Mice were shaved at the dorsal neck where intradermal injections and topical applications were applied. Mice were allowed to acclimate to a Plexiglas chamber for at least 30 min before testing and received intradermal injection of pruritogens (histamine, 500 μg/50 μl; 48/80, 100 μg/50 μl; ET-1, 25 ng/50 μl; chloroquine, 200 μg/50 μl) or saline through a 30-gauge needle into the nape of neck to elicit scratching behavior. After injection, mice were immediately placed back in the chamber, and the scratching behavior was recorded by a Panasonic video camera for a 30-min observation period. Hind limb scratching behavior directed toward the shaved area at the nape of neck was observed. One scratch is defined as a lifting of the hind limb toward the injection site and then a replacing of the limb back to the floor, regardless of how many scratching strokes take place between those two movements. Behavioral analysis was conducted by observers blinded to genotype.

To investigate the topical effects of the specific TRPV4 inhibitor GSK205 or the specific MEK inhibitor U0126 on pruritogen-induced scratching behaviors, mice received a transdermal-topical application of 100 μl of formulated GSK205 (100 μM) or U0126 (0.1 mg/ml) on the shaved area at the nape of neck 20 min before pruritogen injections. Control animals received the same volume of placebo.

Keratinocytes Culture and Ca^{2+} Imaging—Primary mouse keratinocytes were cultured following previous protocol (10). The epidermis from the back skin of newborn WT mice (P0-P2) was separated from the dermis by floating the skin on 0.25% trypsin (Gibco) for 14–18 h at 4 °C. Basal keratinocytes were separated from the cornified sheets by filtration through a 70 μM cell strainer (BD Biosciences). Keratinocytes were plated on collagen-coated dishes or glass coverslips and grown in EME media (Gibco) supplemented with bovine pituitary extract and epidermal growth factor, 10% chelexed fetal bovine serum (Gibco), 100 pmol of cholera toxin (Calbiochem), and 1× antibiotics/antimycotics (Gibco) in an incubator at 5% CO_2 and 37 °C.

Primary human keratinocytes were cultured as previously described (34). In brief, surgically discarded foreskin samples, obtained from Duke Children's Hospital in accordance to institutionally approved IRB protocol, were incubated with Dispase

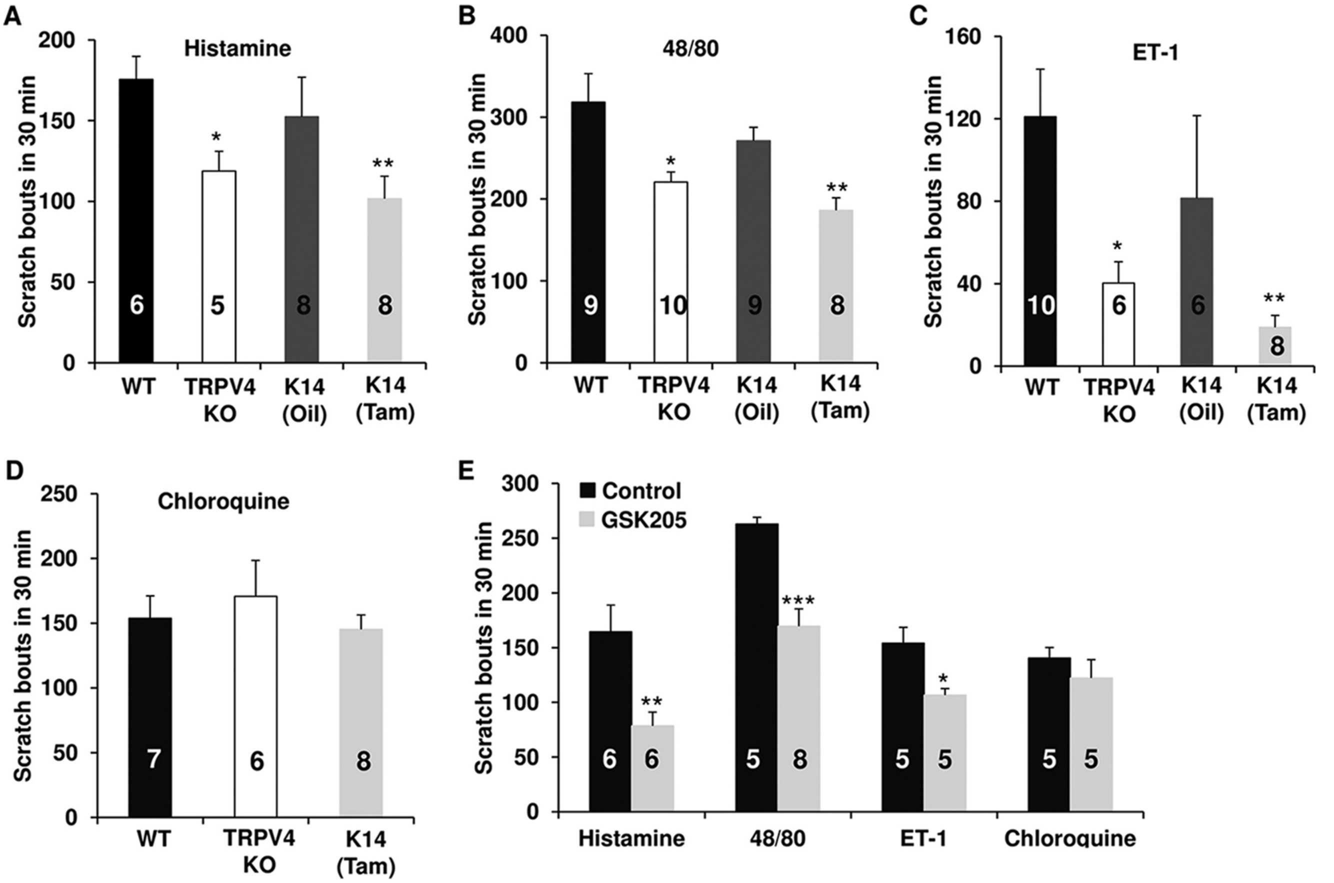

FIGURE 1. ***Trpv4* in skin keratinocytes is essential for histamine-dependent itch.** Histamine (*A*), compound 48/80 (*B*), and ET-1 (*C*), but not chloroquine (*D*), evoked acute scratching behaviors that were significantly attenuated in *Trpv4* cKO (K14-Tam) and pan-null mice (TRPV4 KO) *versus* their respective controls (*A–D*, *, $p < 0.05$; **, $p < 0.01$ *versus* WT). Mice topically transdermally treated with the TRPV4-selective inhibitor GSK205 showed a significant reduction of scratching behaviors (*E*, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus Control*). One-way analysis of variance with Tukey's post hoc test was used for *A–D*, and two-tail *t* test was used for *E*. Group size is indicated in the *bars*.

(Gibco, 4 units/ml) for 12–16 h at 4 °C followed by 0.05% trypsin (Gibco) for 10–20 min at 37 °C. Cells were grown and passaged in keratinocyte serum-free media (Invitrogen) at 37 °C with 5% CO_2 and used at passage 2–3.

Ca^{2+} imaging of primary epidermal keratinocytes in response to chemicals was conducted after loading with 2 μM fura2-AM (Invitrogen) for 30 min after a ratiometric Ca^{2+}-imaging protocol with 340/380-nm blue light for dual excitation. Ratios of emissions were acquired at 0.5 Hz. $\Delta R/R_0$ was determined as the fraction of the increase of a given ratio over baseline ratio divided by baseline ratio.

To investigate the effects of the specific TRPV4 inhibitors GSK205 or GSK219 on pruritogen-induced Ca^{2+} influx and pERK expression, cells were incubated with GSK205 or GSK219 for 15 min before stimulation. Control cells received the same volume of vehicle.

Western Blot—Routine procedures were followed (10, 25, 26, 35). Briefly, cultured keratinocytes and dissected dorsal skin (0.5 × 0.5 cm, the area that received the treatment) were protein-extracted in radioimmunoprecipitation assay (RIPA, Sigma) buffer and electroblotted to nitrocellulose membranes after gel separation of proteins in a 4–15% polyacrylamide gel (Bio-Rad). Membranes were blocked with 5% BSA (Sigma) in TBST, and pERK and ERK were specifically detected with primary antibodies (rabbit anti-pERK (catalog #9101) and anti-ERK (catalog #4695), both at 1:2000; Cell Signaling Technology), secondary antibody (anti-rabbit peroxidase-conjugated, 1:5000; Jackson ImmunoResearch), and chemiluminescence substrate (ECL-Advance, GE Healthcare). Abundance was quantified using ImagePro Plus software. β-Actin, as a control, was detected with a mouse monoclonal anti-β-actin antibody (1:4000; catalog #sc-47778, Santa Cruz) or a rabbit polyclonal anti-β-actin antibody (1:4000; catalog #A5316, Sigma). Immunoblot band intensity was quantitated using the software Image J (National Institutes of Health).

Statistical Analysis—All data are expressed as the mean ± S.E. Two-tailed *t* tests or one-way analysis of variance followed by Tukey's post hoc test were used for group comparisons. $p <$ 0.05 indicated statistically significant differences.

Results

Trpv4 in Skin Keratinocyte Is Critical for Histaminergic Itch—To assess the contribution of keratinocyte TRPV4 channels to acute itch, we subjected *Trpv4* cKO mice to intradermal injections of both histaminergic and non-histaminergic pruritogens. Throughout, we also challenged *Trpv4* pan-null mice in order to be able to compare any eventual behavioral phenotype present in *Trpv4* cKO mice with that in the respective pan-null mouse. All histaminergic pruritogens including ET-1 evoked a solid scratching response, namely histamine itself (Fig. 1*A*), the

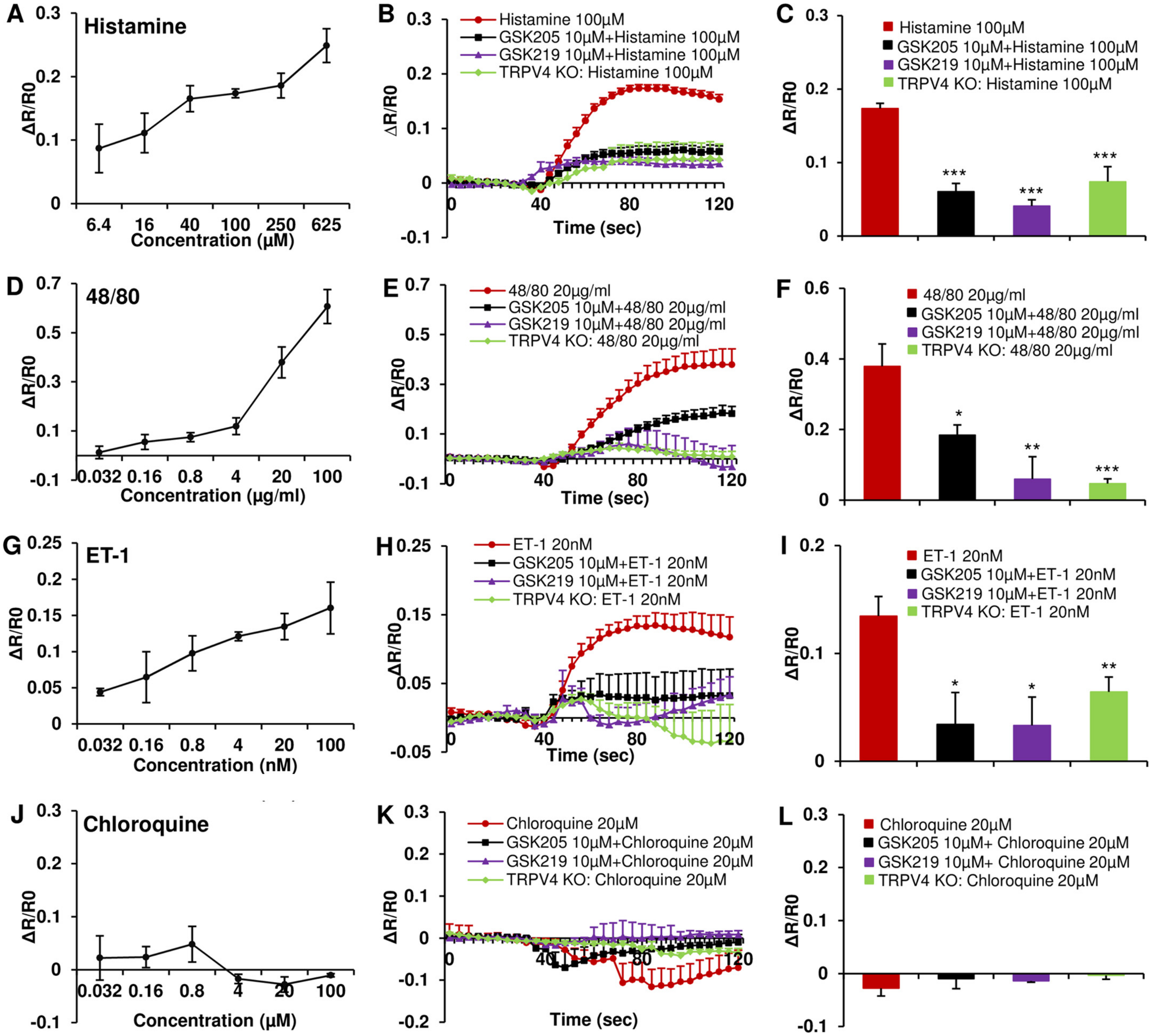

FIGURE 2. **Histamine-dependent pruritogens evoke Ca^{2+} influx in cultured keratinocytes via TRPV4 channels.** Histamine-dependent pruritogens evoke Ca^{2+} influx in cultured keratinocytes via TRPV4 channels. Histamine (*A*), compound 48/80 (*D*), and the partial histaminergic ET-1 (*G*), but not chloroquine (*J*), triggered Ca^{2+} influx in a dose-dependent manner in keratinocytes. The evoked Ca2^{2+} signal was attenuated in cells pretreated with GSK205 or GSK219, both TRPV4-selective inhibitors, and also in cells from *Trpv4*$^{-/-}$ mice (*B* and *C*, *E* and *F*, *H* and *I*, and *K* and *L*; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* the respective pruritogens). Two-tail *t* test was used for statistic analyses. n = 150–300 cells/treatment.

polymeric secretagogue and MrgprX2 activator, compound 48/80 (36–40) (Fig. 1*B*), and the partial histaminergic ET-1 (13, 16, 40–43) (Fig. 1*C*), as did the non-histaminergic chloroquine (40, 44–46) (Fig. 1*D*). The scratching responses evoked by histaminergic pruritogens were significantly attenuated in *Trpv4* cKO mice, most robustly for ET-1. In contrast, scratching in response to chloroquine was not (Fig. 1, *A–D*). This means that TRPV4 ion channels in skin keratinocytes powerfully control organismal itch-related scratching behavior by converting the epidermal keratinocyte into an itch-generator cell that directly or indirectly signals to peripheral pruriceptor sensory neurons. In keeping with this new basic concept, *Trpv4* pan-null mice had a similar profile (Fig. 1, *A–D*), their reduced scratching in response to histaminergic pruritogens, indicating that genetically encoded pan-organismal absence of *Trpv4* renders these mice less sensitive to histaminergic pruritogens. We also topically applied TRPV4-specific small-molecule inhibitor, GSK205 (10, 33), to mouse epidermis. As a result, histaminergic, but not non-histaminergic, scratching was significantly attenuated (Fig. 1*E*), reiterating the conclusion derived from *Trpv4* cKO mice, namely that TRPV4 channels in epidermal keratinocytes are significant molecular actuators of organismal itch, driving the keratinocyte as itch generator cells. In addition, the experiments with topically applied TRPV4 blocking compound point toward a role for TRPV4 ion channel function as a critical contributor, not only reduced expression of the TRPV4 protein.

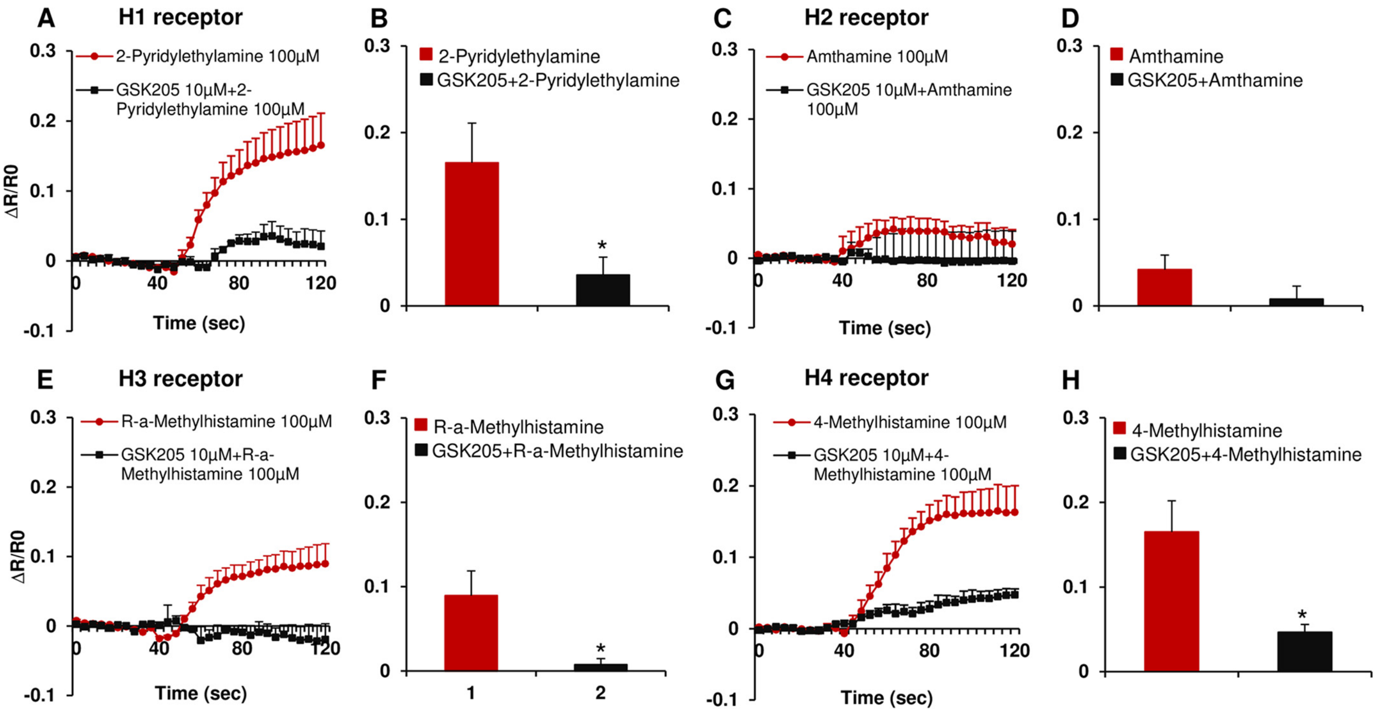

FIGURE 3. **Histamine receptor agonists induce Ca^{2+} influx in cultured murine keratinocytes via TRPV4 channels.** 2-Pyridylethylamine (selective H1 receptor agonist, *A* and *B*), and 4-methylhistamine (selective H4 receptor agonist, *G* and *H*), but not amthamine (selective H2 receptor agonist, *C* and *D*), evoked Ca^{2+} influx in murine keratinocytes, which was attenuated by pretreatment with TRPV4 inhibitor, GSK205. For selective activation of H3 receptor with *R*-α-methylhistamine (selective H3 receptor agonist, *E* and *F*), the increase were less robust. Two-tail *t* test was used for statistic analyses (*B*, *D*, *F*, and *H*; *, $p <$ 0.05 *versus* agonists). n = 100–200 cells/treatment.

TRPV4 Is Required for Histaminergic-dependent Pruritogens-induced Ca^{2+} Influx in Keratinocytes—These *in vivo* results raise the question of how the histaminergic pruritogens that we have used to evoke TRPV4-dependent scratching affect Ca^{2+} signaling in epidermal keratinocytes given that TRPV4 is known to function as a Ca^{2+}-permeable TRP channel in these cells (47). To address this specific question, we stimulated primary murine keratinocytes with the same pruritogens used *in vivo*, then asked whether inhibiting TRPV4 channel activity with a selective small-molecule inhibitor would attenuate any resulting Ca^{2+} transients. We first used the classic pruritogen, histamine, which resulted in a dose-dependent Ca^{2+} signal, strongly attenuated by two selective TRPV4 inhibitors, GSK205 and GSK219 (Fig. 2, *A*–*C*). We obtained a similar reduction of Ca^{2+} signal when stimulating cultured keratinocytes derived from *Trpv4*$^{-/-}$ pan-null mice, confirming the critical role of TRPV4 in Ca^{2+} influx downstream of histamine-receptor signaling. Given the significance of histamine for itch, we also elucidated the receptor subtype present in keratinocytes. Our results suggest histamine receptors of the H1, H3, and H4 subtype to be appreciably involved, not H2 receptors. This finding is in keeping with previously established expression patterns in keratinocytes (48–51) and illustrated in Fig. 3. These three histamine receptor subtypes signaled to TRPV4 as their selective stimulation led to an appreciable Ca^{2+} transient in primary keratinocytes that could be virtually eliminated by GSK205, as also illustrated in Fig. 3. Given the translational medical relevance of this finding, we recapitulated this experiment in primary human keratinocytes. Results are shown in Fig. 4, demonstrating a similar capability of histamine to evoke Ca^{2+} transients in primary human keratinocytes. These Ca^{2+} transients could be completely eliminated with GSK205, as shown in Fig. 4. Our findings indicate the TRPV4-mediated Ca^{2+} signal to rely on H1, H3, and H4 receptors. Congruency of mouse human histamine-TRPV4 signaling suggests an evolutionary conserved allergic inflammation mechanism that underlies integumental signaling from keratinocytes to sensory neurons.

Regarding Ca^{2+} signaling in response to other histaminergic pruritogens, we found that compound 48/80 (Fig. 2, *D*–*F*) and also the partial histaminergic ET-1 (Fig. 2, *G*–*I*) evoked Ca^{2+} transients in mouse primary keratinocytes in a dose-dependent manner that could be blocked with GSK205 and GSK219 or were dramatically reduced in keratinocytes derived from *Trpv4*$^{-/-}$ pan-null mice. Chloroquine, a non-histaminergic pruritogen, however, did not evoke a Ca^{2+} signal in keratinocytes (Fig. 2, *J*–*L*).

Taken together, we detected a TRPV4-dependent Ca^{2+} signal in cultured epidermal keratinocytes in response to histaminergic pruritogens. In view of our *in vivo* findings with *Trpv4* cKO mice, we reason that this Ca^{2+} signal is the cellular signaling correlate of histaminergic pruritogen-activation of TRPV4 channels in epidermal keratinocytes, which co-contributes significantly to scratching behavior *in vivo*.

GSK101, a TRPV4-selective Agonist, Elicits Scratching-behavior Dependent on TRPV4 Expression in Keratinocytes—Having recorded results that suggest (i) the *Trpv4* gene is necessary for scratching behavior in response to histaminergic pruritogens, (ii) TRPV4 channels in epidermal keratinocytes are necessary for these behaviors, and (iii) these channels in epidermal keratinocytes are activated by the G protein-coupled recep-

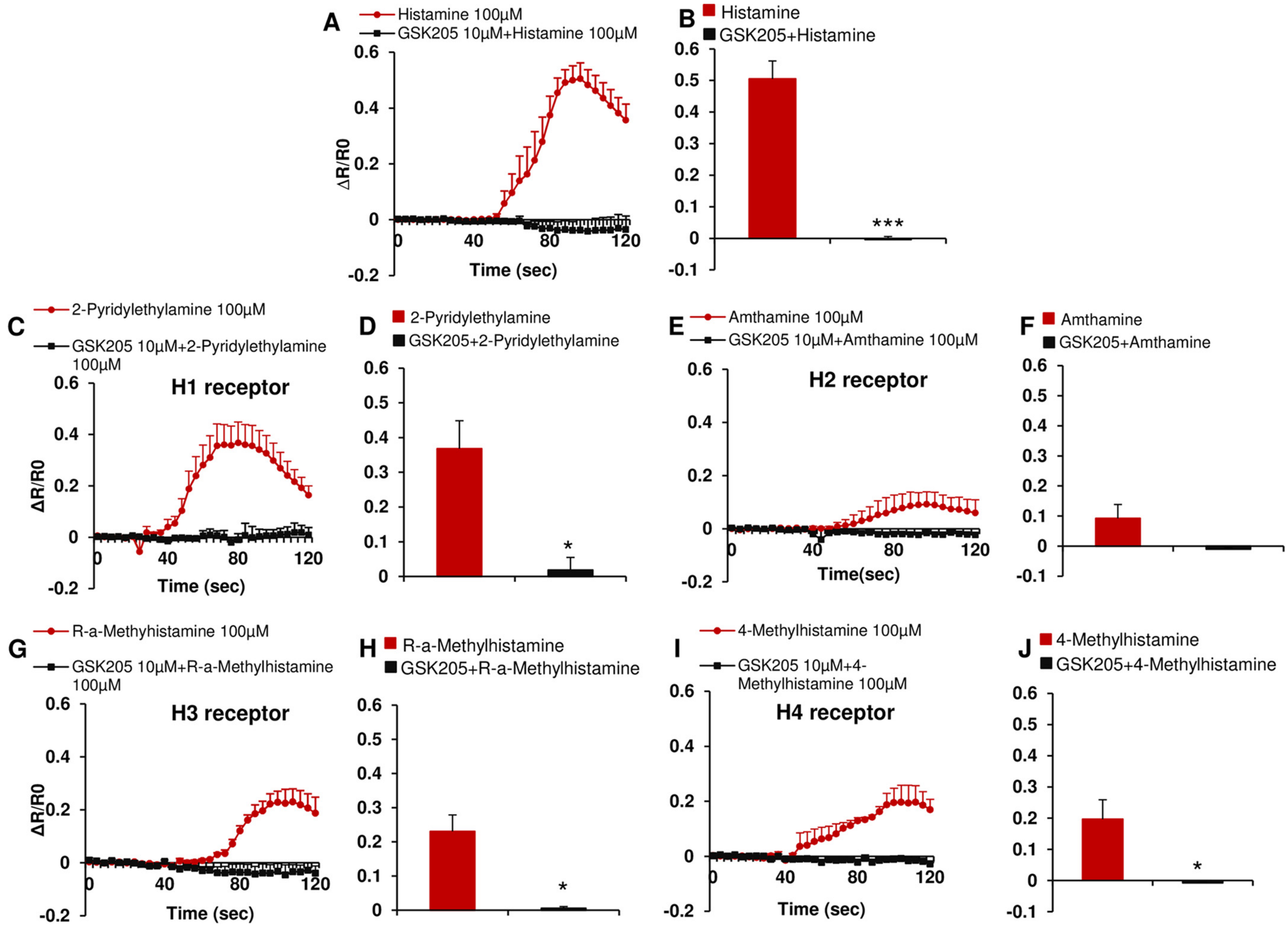

FIGURE 4. **Histamine receptor agonists induce Ca^{2+} influx in cultured human keratinocytes via TRPV4 channels.** Histamine (non-selective H receptor agonist, *A* and *B*), 2-pyridylethylamine (selective H1 receptor agonist, *C* and *D*), *R*-α-methylhistamine (selective H3 receptor agonist, *G* and *H*), and 4-methylhistamine (selective H4 receptor agonist, *I* and *J*), but not amthamine (selective H2 receptor agonist, *E* and *F*), evoked Ca^{2+} influx in a dose-dependent manner in human keratinocytes, which was attenuated by pretreatment with TRPV4 inhibitor, GSK205. A two-tail *t* test was used for statistic analyses (*A*, *D*, *F*, *H*, and *J*; ***, $p < 0.0001$; *, $p < 0.05$ *versus* agonists). n = 100–200 cells/treatment.

tors for the respective pruritogens, we next asked the question of sufficiency of TRPV4 activation for scratching behavior. We documented that increasing concentrations of small-molecule-selective TRPV4 activator, GSK101, evoked itch behavior with increasing frequency (Fig. 5*A*). In a *Trpv4* pan-null knock-out mouse, there was only a marginal, non-significant increase in scratching behavior in response to 65 ng of GSK101 *versus* WT or *Trpv4* pan-null knock-out control, indicating minimal off-target effects of GSK101 as a pruritogen *in vivo*. Thus, scratching behavior, at the organismal level, can be evoked by selective activation of TRPV4. Importantly, we verified that oil-induced *Trpv4* cKO mice scratched not differently from WT mice in response to GSK101. We also showed that *Trpv4* cKO mice induced with tamoxifen showed no significant increase in scratching behavior in response to intradermal injection of GSK101 *versus* WT or *Trpv4* cKO control (Fig. 5*A*). This finding is highly relevant because it suggests that direct activation of TRPV4 channels, expressed by keratinocytes, by intradermal injection of GSK101 leads to scratching behavior in live animals. This is critically dependent on TRPV4 expression by skin keratinocytes. Therefore, we decided next to assess keratinocyte response to selective activation of TRPV4. We did record a dose-response relationship of the resulting Ca^{2+} signal to GSK101 concentrations (Fig. 5*B*). This signal could be significantly attenuated when using two TRPV4-selective inhibitors, GSK205 or GSK219 (Fig. 5, *C* and *D*). Overall, these GSK101-related findings leave open the possibility of a co-contributory role of TRPV4 signaling in sensory neurons or other itch-relevant cells but together with our data, as presented in Figs. 1–4, make the case for an important role for TRPV4 in epidermal forefront signaling as a "pruriceptor-TRP" ion channel in epidermal keratinocytes.

ERK Signaling Downstream of TRPV4 in Skin Keratinocyte Is Essential for Histaminergic Itch—We next addressed the question of what signals intracellularly in epidermal keratinocytes, downstream of TRPV4-mediated Ca^{2+} influx. Choosing a candidate approach, we focused on mitogen-activated protein kinase signaling of the MEK-ERK pathway based on previous results in an epithelial cell type, upper airway respiratory epithelia, that also provides organismal barrier protection in which we demonstrated MEK-ERK activation in response to an environmental irritant (35, 52). We first probed whether there is

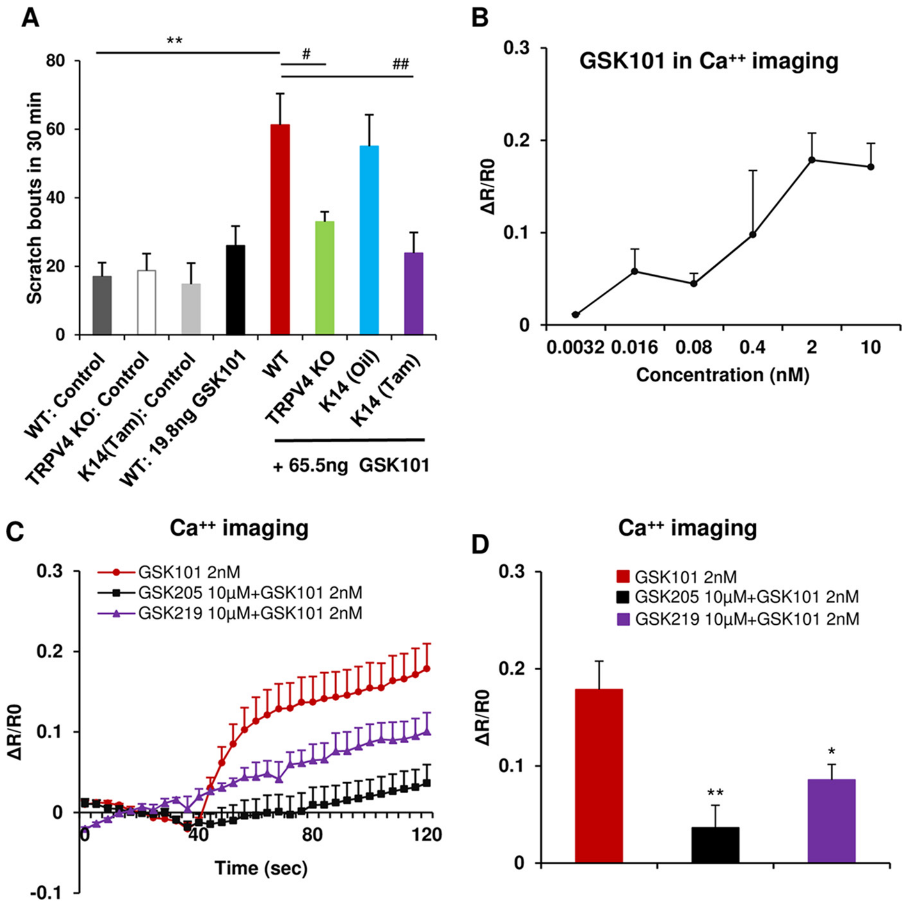

FIGURE 5. **GSK101 elicits scratching behaviors and triggers Ca^{2+} influx in cultured keratinocytes.** Animals injected with the TRPV4-selective activator, GSK101, displayed a significant scratching response that was attenuated in *Trpv4* pan-null mice. Importantly, scratching behavior depended on TRPV4 expression in keratinocytes, evidenced by a complete lack of response to GSK101 in *Trpv4* cKO mice (*A*, **, $p < 0.01$; #, $p < 0.05$; ##, $p < 0.01$). GSK101 evoked a Ca^{2+} response in a dose-dependent manner in keratinocytes (*B*). *C* and *D* illustrate the keratinocyte Ca^{2+} signal evoked by 2 nM GSK101 and its attenuation by TRPV4-selective inhibitors, GSK205 or GSK219 (*, $p < 0.05$; **, $p < 0.01$ *versus* GSK101). One-way analysis of variance with Tukey's post hoc test was used for *A*, and a two-tail *t* test was used for *D*. $n = 4$–5 mice/group (*A*) and $n = 150$–300 cells/treatment (*B*–*D*).

rapid ERK phosphorylation in response to histaminergic pruritogens. We recorded affirmative results at the 10-min time point in cultured keratinocytes and the 30-min time point in skin (dermis-epidermis) for all three histaminergic pruritogens tested, not for non-histaminergic pruritogen chloroquine (Fig. 6). The level of total ERK in both cultured cells and skin was not altered (data not shown). We then next addressed whether this rapid increase in ERK phosphorylation depends on TRPV4 by applying the TRPV4-inhibitor, GSK205. In primary keratinocytes, we observed a complete reversal to non-stimulated levels of pERK. In skin from mice challenged *in vivo*, we detected a similar response (Fig. 6). These findings suggest that pERK activation is down-stream of TRPV4-mediated Ca^{2+} influx. To determine the relevance of MEK-ERK phosphorylation for scratching behavior, we applied a selective inhibitor of MEK, U0126, in a topical formulation to skin. In response to histaminergic pruritogens we observed a significant anti-pruritic effect of topical U0126 when mice were intradermally treated and a lack thereof for non-histaminergic pruritogen, chloroquine (Fig. 7).

Discussion

In this study we describe a novel role for TRPV4 channels in histaminergic itch including ET-1-evoked itch. Importantly, TRPV4 expression and function in epidermal keratinocytes shows a robust contributory role to scratching behavior evoked by histaminergic pruritogens, not for the non-histaminergic chloroquine. This means that keratinocytes of the integument can function as itch generator cells and that TRPV4 plays a significant signaling role in these cells in mediating histaminergic itch. Importantly, direct activation of TRPV4 by intra-dermal injection of TRPV4 activator, GSK101, led to scratching behavior, which critically depended on TRPV4 expression in keratinocytes. This finding underscores the fundamental, hitherto unrecognized role of TRPV4 channels in epidermal keratinocytes in acute histaminergic itch. We recorded complementary findings in primary keratinocyte culture where we observed Ca^{2+} transients in response to the same diverse histaminergic pruritogens that elicit scratching behavior dependent on keratinocyte-TRPV4. We found this Ca^{2+} response to be mediated by TRPV4, which was activated by the respective pruritogens and their cognate keratinocyte-G protein-coupled receptors or directly via selective chemical activator. In epidermal keratinocytes, Ca^{2+} influx via TRPV4 elicits ERK phosphorylation as a downstream signaling event of the forefront pruritogen signaling discovered here. Topical transdermal inhibition of TRPV4 and its downstream kinase target, MEK,

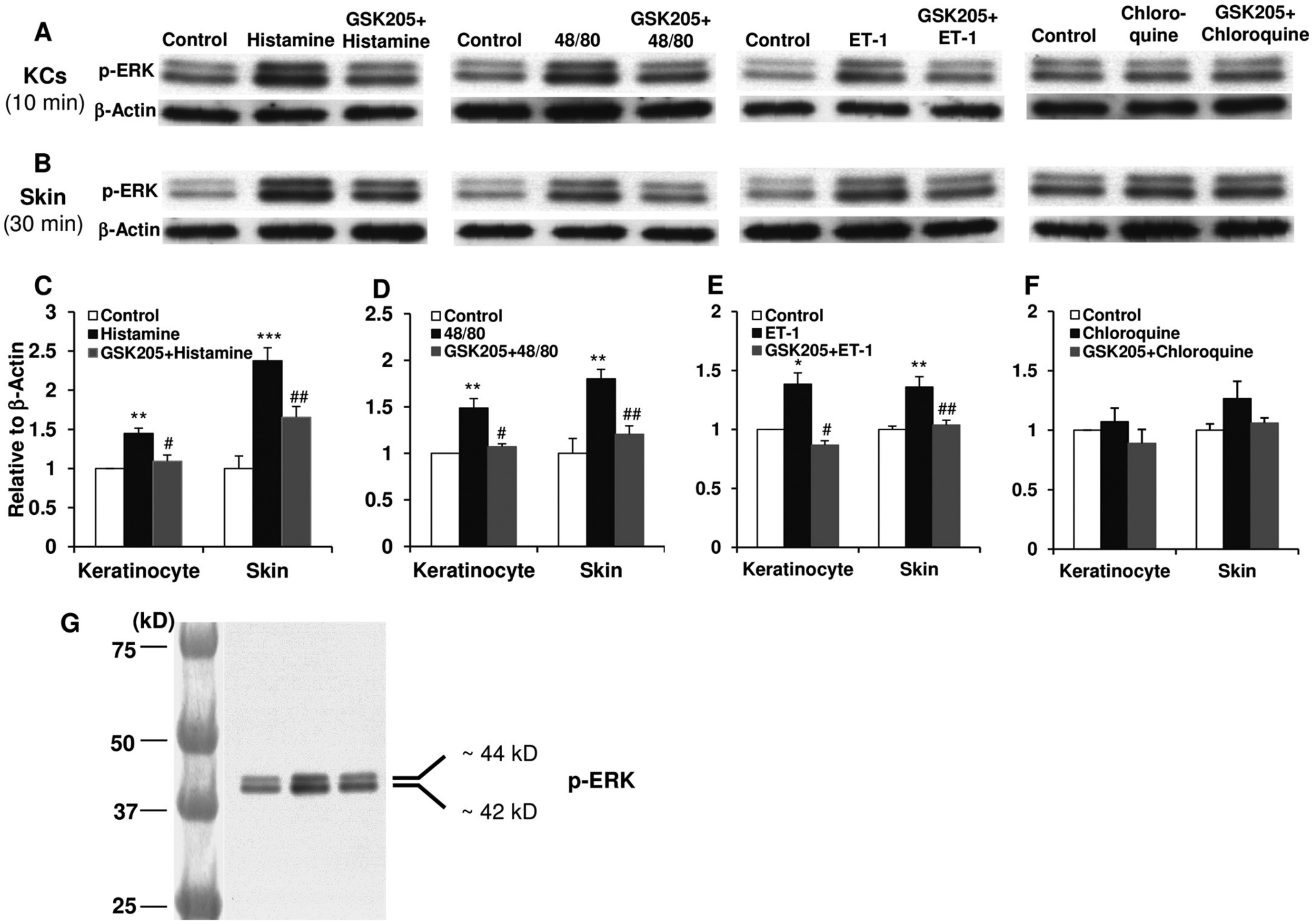

FIGURE 6. ***Trpv4* is essential for the increase of phospho-ERK (*pERK*) in skin keratinocytes (*KCs*) and the integument from live animals in response to histamine-dependent pruritogens.** Western blotting shows pERK expression in cultured keratinocytes (*A*) and nape skin (*B*). *Bar graphs* depict quantitation, demonstrating a significant increase of pERK evoked by histamine (*C*), compound 48/80 (*D*), and ET-1 (*E*), but not chloroquine (*F*) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$: pruritogen *versus Control*). Importantly, a significant increase of pERK depends on TRPV4, demonstrated by its reduction to control levels by pretreatment with TRPV4-selective inhibitor GSK205 (*C–F*: #, $p < 0.05$; ##, $p < 0.01$: GSK205+pruritogen *versus* pruritogen). *G* shows Western blots of pERK with a standard molecular mass marker. Two-tail *t* test was used for statistic analyses. $n = 3$ cultures/group for the cultured keratinocytes and $n = 5\sim6$ mice/group for skin.

which functions upstream of ERK phosphorylation, both showed robust anti-pruritic efficiency in mice challenged with histaminergic pruritogens. Activation of MEK-ERK signaling in keratinocytes is also known from non-itching skin conditions (53, 54). We speculate that MEK-ERK activation by TRPV4 could be the important explanatory difference. This hypothetic concept will have to be tested in future studies. Our results argue for a novel translational medical path of topical treatments to skin that target molecularly defined signaling mechanisms that modulate sensory transduction (55).

In this paper we focus first on acute itch and the role of histaminergic pruritogens. Additional pruritogens need to be studied in the future, with particular focus on chronic itch, a medically relevant condition because of its prevalence and substantial unmet medical need. A central question that we have not addressed in this study is, What specific cellular and molecular mechanisms of cell-to-cell communication do epidermal keratinocytes employ? How does the histaminergic pruritogen-G protein-coupled receptor-TRPV4-Ca^{2+}-pERK pathway evoke these signaling mechanisms, and how does this trigger pruriceptor sensory neurons to transmit the signal toward the nervous system? We hypothesize that soluble factors play such roles, possibly proteins, peptides, small-molecule phospholipids, and lipid molecules that are released from keratinocytes to affect innervating peripheral nerve endings of pruriceptor neurons perhaps either via direct keratinocyte nerve fiber signaling or rather indirectly via involvement of immune, vascular, and other adjacent cells.

A recent study examined the response of spinal cord dorsal horn relay neurons to intradermal injection of ET-1 (16). The study reports that ET-1-sensitive neurons respond to multiple modalities yet that >50% respond to spinal superperfusion of the peptide bombesin, which can activate spinal gastrin-releasing peptide receptors known to function in itch circuits, thus identifying these neurons as part of a specific itch circuit that relies on gastrin-releasing peptide for transmission. When viewing these results together with our current findings, an interesting formation of an ET-1-responsive itch circuit emerges that has its origin with ET-1-responsive keratinocytes that use TRPV4 as critical Ca^{2+} influx mechanism in response

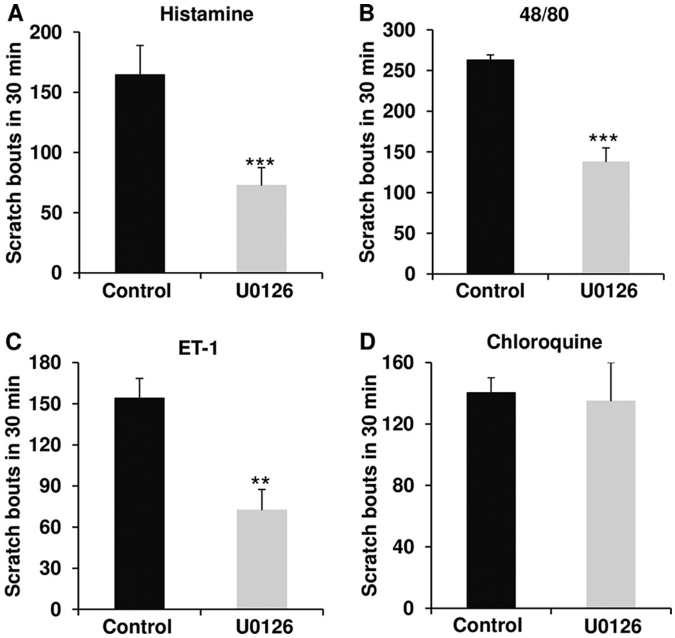

FIGURE 7. **ERK signaling downstream of TRPV4 is relevant for histaminergic pruritogen-evoked scratching behaviors.** Histamine (*A*), compound 48/80 (*B*), ET-1 (*C*), but not chloroquine (*D*) cause scratching behaviors that are significantly attenuated in mice topically pretreated with the MEK selective inhibitor U0126 (**, $p < 0.01$; ***, $p < 0.001$ *versus Control*). Two-tail *t* test was used for statistic analyses. $n = 5–6$ mice/group.

to ET-1 receptor-A activation (10). Subsequently these ET-1-responsive keratinocytes activate innervating peripheral sensory neurons, which need to be more precisely defined in future studies, which in turn relay to spinal cord dorsal horn neurons, more than half specifically dedicated to itch-relay via neurotransmission that relies on gastrin-releasing peptide (16).

In another recent study from these same authors, *Trpv4* pan-null mice were reported to scratch less in response to intradermal injection with serotonin (56). The authors report that they did not see different scratch behavior in response to histamine. In this respect, their results differ from our results in *Trpv4* pan-null mice. This discrepancy may be related to technical detail such as difference in doses of pruritogen, animal ages, and behavioral assessment methods. Of note, the originating line of mice used is identical between our current and the referenced study. We believe that this seemingly perplexing discrepancy can possibly be resolved in future studies that focus on the influence of genetic background on nocifensive and pruritic behavior and, more importantly, on the impact that epigenetic regulation might play. Of note, different phenotypes of identical lines of *Trpv4* pan-null mice, propagated in different laboratories, have been reported previously (57, 58). Importantly, we want to stress that the focus of our present investigation is the distinct contribution of TRPV4 channels in keratinocytes to histaminergic itch, a subject of basic science and translational-medical relevance that is not directly approached in Akiyama *et al.* (56).

With TRPV4 expression in the primary sensory neuron and in the CNS in neurons and glial cells established, which roles do neural and neuronal TRPV4 play in itch transduction, transmission, and plasticity? Whereas these questions remain to be answered in future studies, we wish to reiterate the key concept of TRPV4 as forefront pruriceptor TRP channel functioning in epidermal keratinocytes, to drive the organismal scratch response. This concept bears the translational-medical mandate, as mentioned, to develop selective anti-TRPV4 treatments that can be applied topically and that will also have to be inert regarding epidermal cell growth regulation in view of recent findings of attenuated TRPV4 expression in skin epithelial malignancies (59).

Pruriceptor-TRPs comprise TRPA1, TRPV1, TRPV3 (40, 60–68), and now also TRPV4. Their possible mechanisms of interaction and the respective cellular locale will be attractive subjects for the following chapters in this intriguing story. Possibly, human genetic variation in the respective *TRP* pruriceptor genes might be relevant for different itch susceptibilities both for physiologic and pathologic forms of itch.

Author Contributions—Y. C. and W. B. L. conceived and coordinated the study and analyzed the data. Y. C., Q. F., and Z. W. designed, performed, and analyzed the experiments. J. Y. Z. contributed reagents. Y. C., J. Y. Z., A. S. M., R. P. H., and W. B. L. drafted and revised the article. All authors reviewed the results and approved the final version of the manuscript.

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Hot Topic Review

Pleiotropic function of TRPV4 ion channels in the central nervous system

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Edited by: Peter Raven

New Findings

- **What is the topic of this review?**
  In this concise review, we highlight insights into the role of transient receptor potential, vanilloid type 4 (TRPV4) ion channels in the CNS, results that have been contributed over the last 16 years since the initial discovery of the channel.
- **What advances does it highlight?**
  TRPV4 has been found to function in neurons, astroglia and microglia, both in physiological (e.g. astrocytic neurovascular coupling, neuronal membrane potential at physiological temperature) and in pathological conditions (e.g. mechanical trauma), so far recorded as exciting findings in need of more in-depth mechanistic clarification.

Transient receptor potential, vanilloid type 4 (TRPV4) ion channels are osmo-mechano-TRP channels, with pleiotropic function and expression in many different types of tissues and cells. They have also been found to be involved in pain and inflammation. Studies have focused on the role of TRPV4 in peripheral sensory neurons, but its expression and function in central nervous glial cells and neurons has also been documented. In this overview, based on the senior author's (WL) lecture at the recent recent joint meeting of APS/The Physiological Society in Dublin, we concisely review evidence of TRPV4 expression and function in the CNS and how TRPV4 function can be modulated for therapeutic benefit of neuropsychiatric disorders. Novel TRPV4-inhibitory compounds developed recently in the authors' laboratory are also discussed.

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Introduction

Transient receptor potential, vanilloid type 4 (TRPV4) ion channels (Liedtke *et al.* 2000) are also expressed in cells of the CNS, including neurons, astrocytes and microglia (White *et al.* 2016). Cell physiology experiments indicate that TRPV4 channels can function as Ca^{2+}-permeable cation channels that are gated by various stimuli, such as cell swelling, low pH, mechanical stress and temperature (Liedtke & Kim, 2005; Liedtke, 2008; Guilak *et al.* 2010; McNulty *et al.* 2015; White *et al.* 2016). Whether this currently established functional profile is particularly relevant for the role that TRPV4 plays in the CNS remains to be determined. As of today, 16 years after its initial description (Liedtke *et al.* 2000; Strotmann *et al.* 2000), studies on specific functions of TRPV4 in various regions of the CNS are not further advanced than the early stages of exploration. Slow progress could be attributable to low levels of TRPV4 expression in certain cell types within the CNS, yet still contributing to important function. TRPV4 could function both developmentally and postdevelopmentally as a sensor-signalling molecule. Despite this lack of a clear picture, TRPV4 appears to function in glial cells and neurons in basic physiological

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and in specific pathological conditions, which are reviewed concisely here. One particular focus of this article is to relate known findings on TRPV4 in the CNS to what this might mean for medicinal translational purposes.

Role of TRPV4 in astrocytes

TRPV4 channels have been found to be expressed in astrocytes. These channels were localized in the plasma membrane of astrocytes and exhibited activation in response to a selective agonist, showing a typical outwardly rectifying cation current (Benfenati *et al.* 2007). As a Ca^{2+}-permeable channel, TRPV4 was shown to influence neurovascular coupling through Ca^{2+}-induced Ca^{2+} release from inositol trisphosphate receptors in astrocytic endfeet (Fig. 1*A*; Dunn *et al.* 2013). Studies indicate that astroglial cells can modulate neuronal excitability in the hippocampus, cortex and hypothalamus and that TRPV4 might function as a key player in this excitation (Simard & Nedergaard, 2004; Shibasaki *et al.* 2014). In the hippocampus, for example, TRPV4 is highly expressed in astrocytes of the CA1 region, and its enhanced expression in this region coincides with the development of astrogliosis (Shirakawa *et al.* 2010; Butenko *et al.* 2012).

Other studies suggest that TRPV4 may form a complex with aquaporin-4 (AQP4/TRPV4) and function in control of cell volume in astrocytes and be involved in regulating interstitial tonicity in the brain, as well as in formation of brain oedema (Benfenati *et al.* 2011). Furthermore, TRPV4 channels in hippocampal astrocytes were considered to function in oxidative stress-induced cell damage (Bai & Lipski, 2010). Other evidence suggests that astrocytic TRPV4 may be involved in neuronal toxicity evoked by the Alzheimer's disease-associated peptide, $A\beta_{40}$ (Fig. 1*C*; Bai & Lipski, 2014). Astrocytes may therefore play crucial roles in the homeostatic regulation of the CNS not only in physiological but also in pathological conditions. TRPV4 channels expressed in astroglial cells were also shown to mediate infrasound-induced neuronal injury (Fig. 1*B*), a model for blast-induced traumatic brain injury, impairing learning and memory in rats (Shi *et al.* 2013). This last study also indicated that injury was more pronounced in the hippocampal CA1

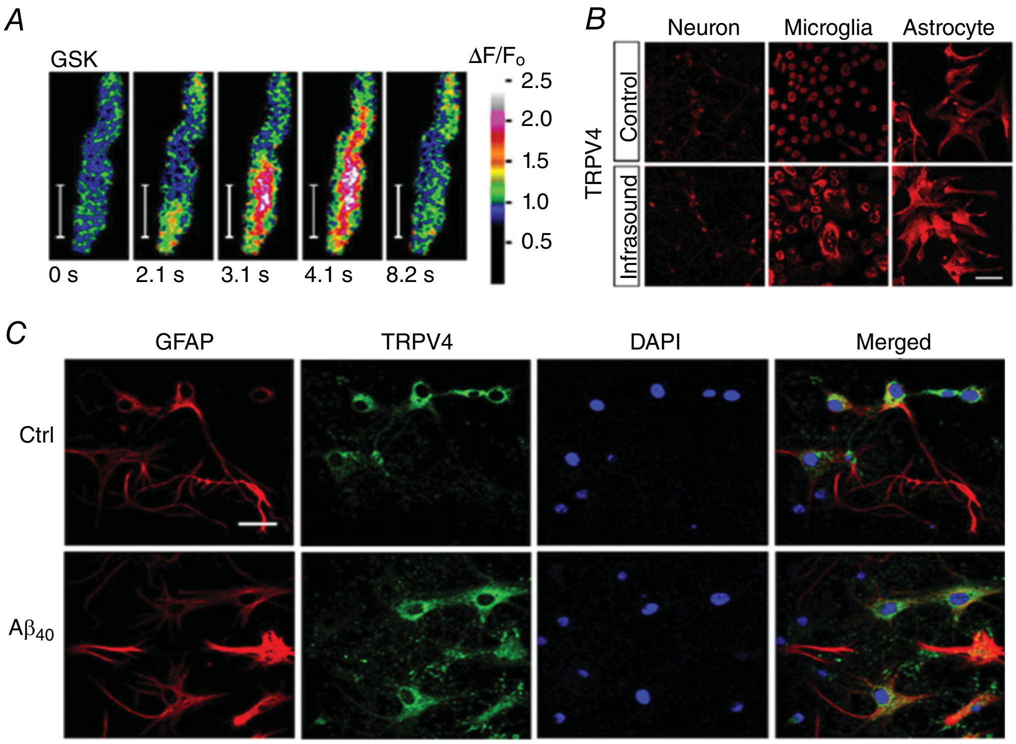

Figure 1. TRPV4 in astrocytes in physiological conditions and upregulated in response to injury
A, time course of an astrocytic endfoot Ca^{2+} transient evoked by selective stimulation of TRPV4, using selective activator compound GSK101; from Dunn *et al.* (2013). *B*, infrasound-induced mechanical stress response of cultured CNS lineages; note upregulation of TRPV4 in response to infrasound in microglia and astrocytes; from Shi *et al.* (2013). *C*, exposure of cultured astrocytes to Alzheimer's disease-associated amyloid peptide $A\beta_{40}$ induces increased expression of astrocytic intermediate filament protein, GFAP, and TRPV4 ion channels; from Bai & Lipski (2014).

region, where astrocytic and microglial activation was observed prior to neuronal apoptosis. Inhibition of TRPV4 protected neurons from infrasound injury by decreasing the expression levels of glial cell-released pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-α.

Role of TRPV4 in microglia

Interestingly, microglial activation after an injection of lipopolysaccharide into the mouse cerebral ventricle was inhibited by concurrent administration of a TRPV4 agonist, 4α-phorbol 12,13-didecanoate (Shirakawa *et al.* 2010; Konno *et al.* 2012). Shirakawa *et al.* (2010), Konno *et al.* (2012) and Shi *et al.* (2013) describe seemingly opposing roles of TRPV4 in microglial activation in response to mechanical stimulation *versus* chemical stimulation via lipopolysaccharide, indicating the important but complex role of TRPV4 in microglia, which is currently underexplored, thus in need of future study.

Role of TRPV4 in CNS neurons

In hippocampal neurons, the influx of cations through TRPV4 channels at physiological temperature may control neuronal excitability by regulation of the resting membrane potential (Shibasaki *et al.* 2007; Shibasaki *et al.* 2015*a*; see also Shibasaki *et al.* 2015*b*), and possibly also in other neurons, with wide implications for brain functioning, manifested in several abnormal behavioural parameters in *Trpv4*$^{-/-}$ mice, as recently documented (Shibasaki *et al.* 2015*a*). It is possible that TRPV4 expression and function in neurons might play a significant role in seizures. In larval zebrafish, for example, febrile seizure-related neural activity, which was triggered by an increase in brain temperature, was shown to be blocked by a TRPV4 antagonist and not by GABA re-uptake inhibitors (Hunt *et al.* 2012). Furthermore, increased TRPV4 expression in cortical lesions of patients with focal cortical dysplasia, a known form of therapy-refractory epilepsy, indicates that TRPV4 might contribute to cortical malformation and maldevelopment, which may facilitate epileptogenesis (Chen *et al.* 2016). As an important qualifier, indicative of the superficial level of inquiry in this field, changed expression levels do not conclusively substantiate any association with disease pathogenesis. In other studies, mutations in *TRPV4*, hereditary TRPV4 channelopathies, were shown to cause excessive Ca^{2+} influx related to motor nerve axonopathy and spinal muscular atrophy (Fecto *et al.* 2011; Jang *et al.* 2012). Although the human genetics of these disorders are clear, it is unclear why some *TRPV4* channelopathy mutations cause skeletal malformations, whereas other mutations detrimentally affect spinal motoneurons. For the former, mechanistic evidence regarding a feasible pathomechanism has been provided, at least an initial inroad (Leddy *et al.* 2014*a*,*b*). In contrast, for the sequence of events between excess Ca^{2+} influx and subsequent motoneuron dysfunction, this has hitherto remained elusive.

Interestingly, in ageing, age-related expression changes of TRPV4 have been reported in pyramidal cortical neurons, thalamus, basal nuclei of the cerebellum and in the spinal cord (Lee & Choe, 2014). The expression of TRPV4 in neurons in these brain regions could be the basis for (mal)function of TRPV4 in pathological conditions. For example, findings by Lee *et al.* (2012) on age-dependent expression of TRPV4 channels may be indicative of the role of TRPV4 in the pathogenesis of age-related neurodegenerative diseases. Neuronal TRPV4 channels may therefore be an important therapeutic target for cognitive, motor and ageing-related disorders. TRPV4 inhibitors have been described and were recently described as orally available compounds (Jia *et al.* 2004; Krause *et al.* 2005; Phan *et al.* 2009; Vincent *et al.* 2009; Morty & Kuebler, 2014; Feetham *et al.* 2015; Qi *et al.* 2015) and also as 'dual inhibitors' of TRPV4 and TRPA1 (Kanju *et al.* 2016), which might be an attractive possibility given the postulated function of astrocytic TRPA1 (Lee *et al.* 2012; Shigetomi *et al.* 2012, 2013; Wei *et al.* 2016). Such inhibitors, if they are intended for oral use, will have to pass through the blood–brain barrier (although, for example, the blood–brain barrier is more penetrable in certain CNS disorders, such as multiple sclerosis). As an alternative, if TRPV4 or TRPV4/TRPA1 (dual) blockers can potently treat more severe neuropsychiatric illnesses safely and effectively, intrathecal administration remains a plausible alternative (e.g. for therapy-refractory high-intensity chronic pain, note its treatment with intrathecal ziconotide; McDowell & Pope, 2016).

Although an interesting topic, the role of TRPV4 in the CNS in systemic osmoregulation and hydromineral regulation is not reviewed here (but see Liedtke & Friedman, 2003; Janas *et al.* 2016), mainly because it was covered in a separate presentation at the recent joint meeting of APS/The Physiological Society in Dublin, Ireland, and therefore not covered in the overview talk by the senior author (WL). This qualifier also applies to the important topic of the role of TRPV4 in the CNS in the choroid plexus (Takayama *et al.* 2014), first described by Liedtke *et al.* (2000).

Conclusion

In this concise review, we highlight the important, yet currently underexplored role of TRPV4 in the CNS in glial cells and neurons, which has so far not been met with appropriate attention and matching investigative scrutiny.

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Additional information

Competing interests

None declared.

Author contributions

P.K. and W.L. conceived the idea to write this paper and wrote it together.

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